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Immunology of Cultivated Mammalian Cells. I. Species Specificity Determined by Hemagglutination [1, 2]

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SUMMARY

Guinea pigs were immunized with human cell strains EE and HeLa, rabbit cell strains CRE and ERK-1, and tertiary cultures of domestic rabbit and rhesus monkey kidney. Antiserums to CRE and rabbit kidney cells agglutinated rabbit but not human erythrocytes. Antiserums to EE, HeLa, and ERK-1 cells agglutinated human but not rabbit erythrocytes. Induced hemagglutinins were distinguished from natural heterophil hemagglutinins and blood group-specific antibodies by absorption of serums with cells or erythrocytes and by agglutination inhibition with whole or disrupted cells or erythrocytes. Guinea pigs hyperimmunized with cells within the groups (*a*) EE, HeLa, and ERK-1 and (*b*) CRE and rabbit kidney exhibited anamnestic courses of hemagglutinin production; hyperimmunization between groups produced primary responses to secondary immunization. Guinea pigs injected with 1000 : 1 mixtures of EE and CRE cells produced hemagglutinins for rabbit as well as human erythrocytes. Antiserums to human and ERK-1 cells contained some induced agglutinins for rhesus monkey erythrocytes; antiserums to rhesus kidney cells contained agglutinins for rhesus and cynomolgus erythrocytes, but not human erythrocytes. Antiserums to calf-serum culture medium agglutinated calf erythrocytes weakly and irregularly. Antiserums contained no induced agglutinins for erythrocytes of other species.—J. Nat. Cancer Inst. 24 : 1007–1019, 1960.

AN UNEQUIVOCAL test for species identity of mammalian cells in continuous culture is desirable. A preliminary report (*1*) and this paper describe the species reactivity of human cells of normal and malignant origin in continuous culture, rabbit cells of normal origin in continuous and tertiary culture (that is, second passage from tissue suspensions), and monkey cells in tertiary culture, as revealed by agglutination of various erythrocytes by anticellular guinea-pig serums.

In pilot experiments, complement fixation and cytotoxicity tests showed broad interspecies cross reactions. Cross absorption of antiserums with cells was unsatisfactory because removal of cross-reacting components was accompanied by loss of specific reactivity. The complement

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fixation additionally was complicated by acquisition of anticomplementary properties by cell-absorbed antiserums. Agglutination of erythrocytes by anticellular serums was satisfactory because it did not involve reactions between antibodies and inducing antigen complexes, and because the test procedure was readily performed. The Westwood-Porton cell strain ERK-1, derived from embryonic normal rabbit kidney (*2*, *3*), was studied because the cells resembled human cells in their susceptibility to poliovirus infection despite rabbit origin.[3]

MATERIALS AND METHODS

Cultivation and treatment of cells.—Cells used included (*a*) human esophageal epithelial cells of normal origin (Minn. EE 55–12–1), passages 57 to 109 of the parental strain (*5*); (*b*) strain HeLa of human malignant origin, as the parental strain maintained in this laboratory (*5*); (*c*) strain ERK-1 (*3*) of embryonic domestic rabbit kidney cells, propagated from passage 187, supplied by courtesy of Dr. J. C. N. Westwood; (*d*) epithelial cells in continuous culture from normal cottontail rabbit skin (Minn. CRE 57–8–1), passages 35 to 54 of the parental strain originated in this laboratory (*6*); and (*e*) normal domestic rabbit kidney and normal rhesus monkey kidney cells of the second serial passage from primary culture. Cells were grown in yeast extract medium (YEM), as described by Syverton and McLaren (*5*), supplemented with 10 percent each of human and rabbit serum. Before use in experiments, cultivated monolayers were incubated for 2 days at 37° C. under maintenance solution (*7*) supplemented with 10 percent calf serum. Rabbit and monkey kidney primary cultures from trypsinized cell suspensions were established in Hanks' balanced salt solution supplemented with 0.5 percent lactalbumin hydrolysate and 5 to 10 percent calf serum. Subculture was accomplished by trypsinization as usual. Subcultures were washed repeatedly at each feeding to eliminate erythrocytes and tissue fragments carried over from the primary culture. For experimental use, cell monolayers were washed with 0.01 M phosphate-buffered NaCl solution at *p*H 7.0 and detached from glass with a rubber scraper. Cells were dispersed by repeated pipetting, washed 4 times, and dispersed in the salt solution.

Immunization of guinea pigs.—Phosphate-buffered NaCl solution containing dispersed cells was emulsified in a mortar with an equal volume of adjuvant (1 part Arlacel A and 4 parts Bayol F). Guinea pigs (350–400 gm.) received intramuscular injections of 0.25 ml. of emulsion containing 10^6 cells. Animals were bled by cardiac puncture before, and weekly for 4 successive weeks after, receiving an injection. After 5 to 6 weeks, animals received reinjections of other cells than the type used for the first injection (cross hyperimmunization). Blood was obtained before, and weekly for 3 weeks after, the second injection.

[3] These studies were under way when it was learned in this laboratory and elsewhere (*4*) that the chromosomal complement of the ERK-1 cell strain was similar to that of cells of human origin.

Hemagglutination procedure.—Serums were inactivated at 56° C. for 30 minutes. Inactivated serums were diluted one fourth in 0.01 M phosphate-buffered NaCl solution at *p*H 7.0. Serial twofold dilutions were prepared from this dilution and dispensed in 0.2 ml. amounts into cups of plastic trays. Erythrocytes in 0.3 to 0.5 percent (v/v) concentration were added in 0.05 ml. amounts. Trays covered with cellophane sheets were incubated at 37° C. for 4 to 6 hours and agitated gently at half-hour intervals. Diminution of agglutination with serum dilution was gradual rather than sharp. The titer was read as the highest serum dilution giving macroscopically visible erythrocyte aggregates. Several serum or erythrocyte samples were compared in each test, so that relative titers could be determined accurately by matching serum-dilution cups showing the same degree of agglutination.

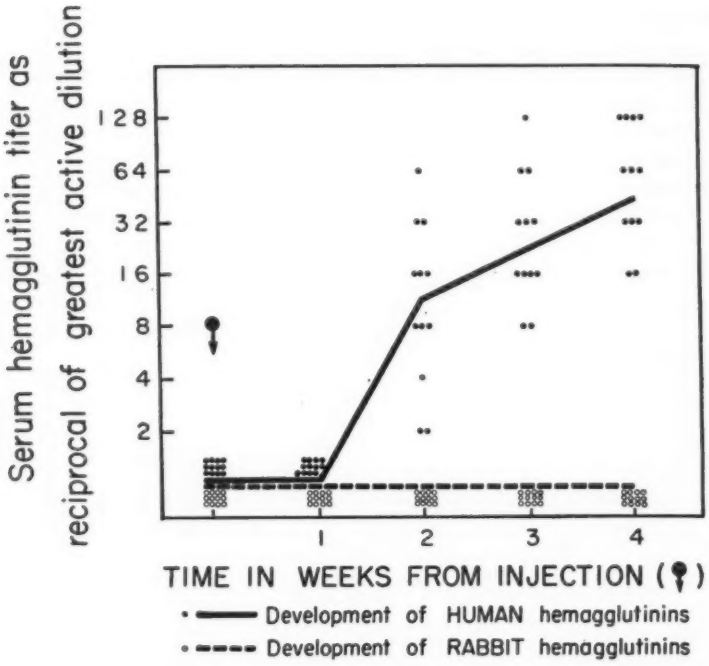
Absorption procedures.—Cultured cells described were ground lightly and washed twice with low-speed horizontal centrifugation (1000 rpm). Sediment obtained from 3 to 5 million cells was mixed with 0.5 ml. inactivated serum in one-fourth dilution in a screw-cap tube, which was rotated at an angle during incubation for 6 to 8 hours at room temperature. The mixture was recentrifuged and the supernatant fluid absorbed similarly overnight. The sediment was removed finally by centrifugation at about 5000 $\times$ g. Absorption for control purposes was effected by use of 2 percent erythrocytes.

Hemagglutination-inhibition procedure.—Cultured cells were disrupted by treatment at 9 kc. per second for 3 minutes in a Raytheon magnetostrictor. Disrupted cells, or whole cells as used for immunization, were dispersed in buffered salt solution at about 10^7 cells per ml.; 0.1 ml. amounts of twofold serially diluted suspension were mixed with 0.1 ml. of diluted serum containing 2 hemagglutination units, according to the final reaction volume of 0.25 ml. After incubation at 37° C. for 4 to 6 hours with repeated agitation, 0.05 ml. of erythrocyte suspension was added to each cup and the hemagglutination titer read after standard incubation. For inhibition controls, 2 percent erythrocyte suspensions lysed with distilled water were used.

RESULTS

Production of Hemagglutinins by Guinea Pigs Immunized With Culture Cells

Groups of 8 to 12 guinea pigs received injections of EE, CRE, second-passage domestic rabbit kidney, and ERK-1 cells; 4 animals received injections of HeLa cells. Time courses of hemagglutination production are shown in text-figures 1 to 3. Similar courses followed immunization with EE and HeLa cells. Injection with EE or CRE cells caused guinea pigs to produce agglutinins for human or rabbit erythrocytes respectively. Formation of rabbit hemagglutinins on the average was weaker than development of human hemagglutinins. Guinea pigs immunized with



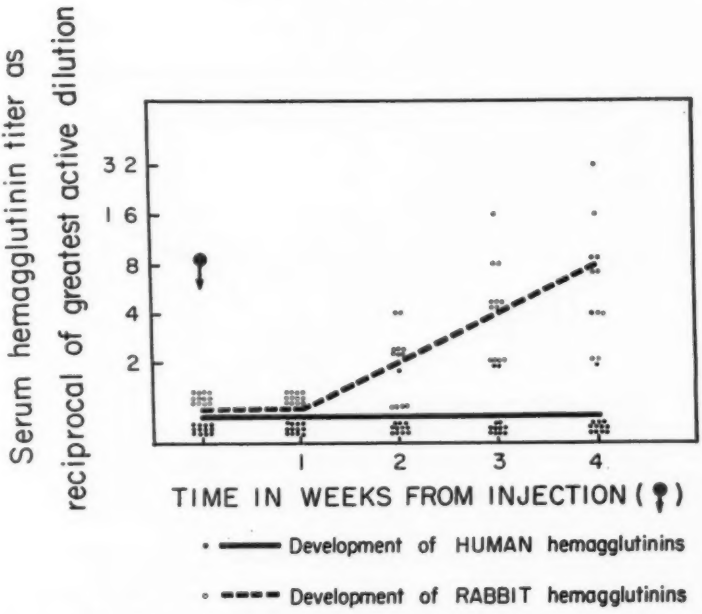
TEXT-FIGURE 1.—Titers of agglutinins for human and rabbit erythrocytes produced in time by guinea pigs receiving intramuscular injections of mineral-oil adjuvant mixed with 10^8 human esophageal epithelial cells in continuous culture (Minn. EE 55-12-1). For this text-figure and those that follow, each point represents the titer of induced hemagglutinins in serum from a single animal, calculated by deduction of dilution steps equivalent to the titer of natural heterophil hemagglutinins in control preimmunization serum. The time regression joins geometric mean titers.

ERK-1 cells (text-fig. 3) responded by strong production of human hemagglutinins, as was characteristic of animals immunized with human EE and HeLa strains. No increase in rabbit hemagglutinins resulted from immunization with ERK-1 cells.

Hemagglutinins Produced in Response to Mixed Immunization

Text-figure 4 shows the time of hemagglutinin production by guinea pigs immunized with EE cells mixed with CRE cells in a ratio of 1000 :1. As seen in comparison with results of text-figures 1 to 3, the cell mixture induced responses individually characteristic of human and rabbit cells.

Independence of the immune responses was confirmed by cross hyperimmunization. As shown in text-figure 5, a significant difference in time to antibody response was observed. Groups of animals receiving injections first of CRE cells and then of rabbit kidney cells showed accelerated production of hemagglutinins expected as an anamnestic immune response. The response to secondary injection of EE cells, however, was delayed as



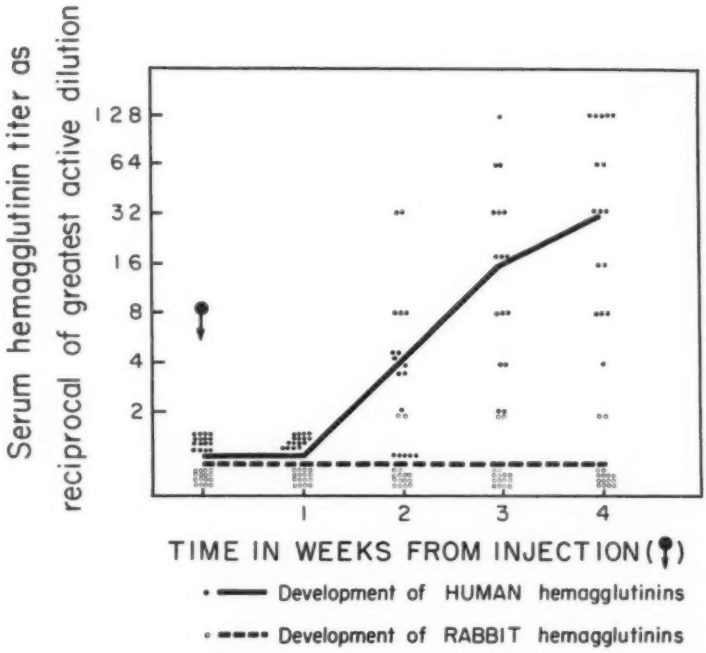
TEXT-FIGURE 2.—Titers of agglutinins for human and rabbit erythrocytes produced in time by guinea pigs receiving intramuscular injections of mineral-oil adjuvant mixed with 10^6 cottontail rabbit epithelial cells in continuous culture (Minn. CRE 57-8-19). Each point represents the titer of induced hemagglutinins in serum from a single animal; the time regression joins geometric mean titers.

expected for a primary response. Responses of the groups of animals receiving injections of EE and CRE cells in sequence similarly were distinct. This cross-hyperimmunization technique related ERK-1 and human EE cells, but not ERK-1 and rabbit cells.

These results suggested that the hemagglutination test as used herein was a highly specific and sensitive indicator of the species of animal cells in culture. Verification was sought in study of the bases of this hemagglutination reaction.

Antigenic Activity of Mediums

ERK-1 cells were grown in medium containing (*a*) 20 percent human serum, (*b*) 20 percent rabbit serum, or (*c*) 10 percent human serum mixed with 10 percent rabbit serum. Cells grown in these mediums were maintained under 10 percent calf serum before use for immunization of groups of 4 guinea pigs. Animals were immunized also with undiluted maintenance medium containing 10 percent calf serum, mixed with the mineral-oil adjuvant. Time courses of human hemagglutinin production by guinea pigs immunized with ERK-1 cells of the different lots did not differ significantly, and no rabbit hemagglutinins were produced. Neither

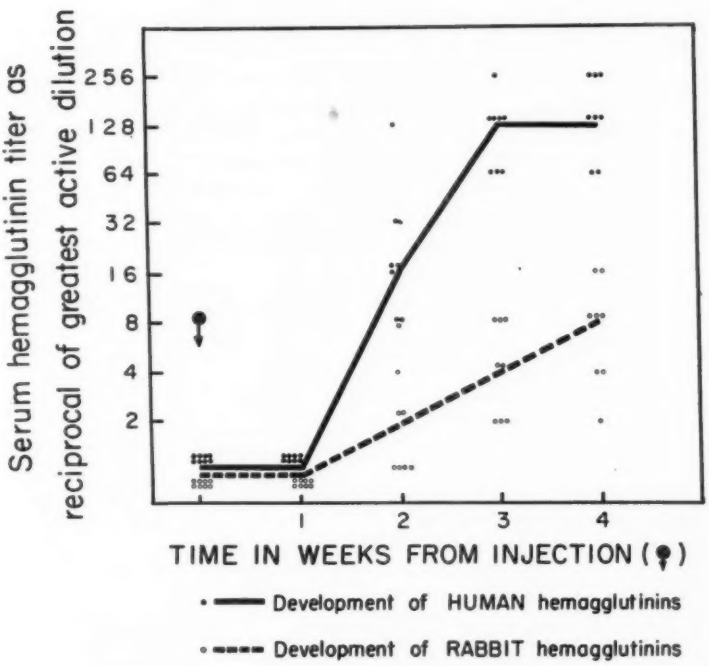


TEXT-FIGURE 3.—Titers of agglutinins for human and rabbit erythrocytes produced in time by guinea pigs receiving intramuscular injections of mineral-oil adjuvant mixed with 10^8 cells of the Westwood-Porton ERK-1 strain. Each point represents the titer of induced hemagglutinins in serum from a single animal; the time regression joins geometric mean titers.

human nor rabbit hemagglutinins developed in response to the maintenance medium.

Specificity of Hemagglutination

Thirteen pairs of serum specimens were tested for agglutination of 8 samples of human erythrocytes, 4 each of domestic rabbit and mouse, 3 each of swine and sheep, 2 each of rhesus monkey, cynomolgus monkey, and chicken, and 1 each of cottontail rabbit, calf, horse, cat, dog, and hamster. Each pair of specimens included serum from preimmunized guinea pigs and from guinea pigs immunized with EE (4 specimens), ERK-1 (5 specimens), or CRE (4 specimens) cells. EE and ERK-1 cells provoked agglutinins for human erythrocytes and to a lesser extent for rhesus erythrocytes. Guinea pigs receiving an injection of second-passage rhesus kidney cells produced agglutinins for rhesus and cynomolgus erythrocytes. CRE or second-passage rabbit kidney cells provoked agglutinins for domestic and cottontail rabbit erythrocytes only. Control serums from guinea pigs injected with calf-serum maintenance medium agglutinated calf erythrocytes weakly and irregularly. Tests of antiserums



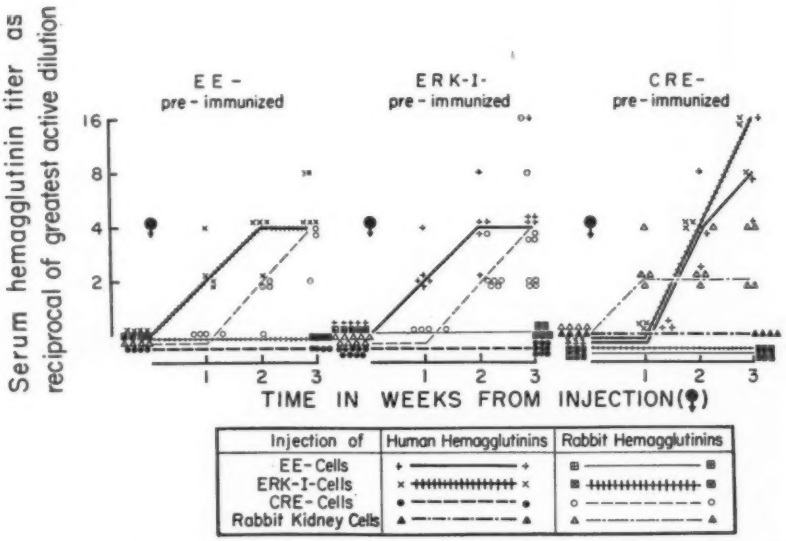
TEXT-FIGURE 4.—Titers of agglutinins for human and rabbit erythrocytes produced in time by guinea pigs receiving intramuscular injections of mineral-oil adjuvant mixed with 10^8 human EE and 10^9 rabbit CRE cells. Each point represents the titer of induced hemagglutinins in serum from a single animal; the time regressions join geometric mean titers.

failed to yield significant evidence of induced hemagglutinins for erythrocytes of remaining species.

Differentiation of Natural and Induced Hemagglutinins

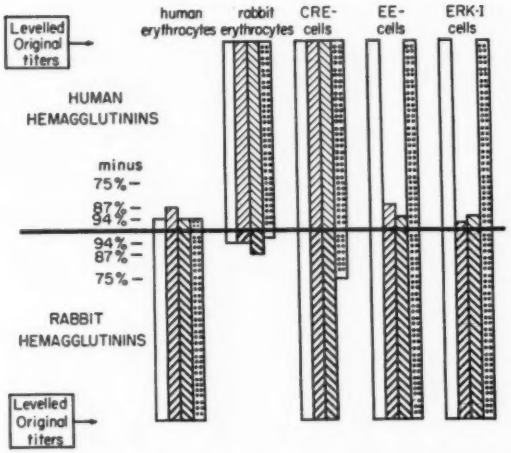
Before immunization, all guinea-pig serums contained agglutinins to varying titer for erythrocytes of almost all species tested. Some serums exhibited high titers with human, rabbit, monkey, sheep, and even cat erythrocytes; titers with calf, swine, and chicken erythrocytes commonly were low or insignificant. Presence of heterophil hemagglutinins necessitated evaluation of the time course of hemagglutinin production by each immunized animal, beginning with the preimmunization period. Relative activity of newly formed hemagglutinins was estimated as a number of twofold dilution steps by which agglutinating activity increased over the natural titer. Although this measure could not be regarded as quantitative, because titers of natural agglutinins varied widely with animals, it was useful for comparisons between serums. The first question was whether the increasing hemagglutinin content of guinea-pig serums

after immunization represented a nonspecific rather than a specific response. Specificity of response was indicated by experiments such as those illustrated in text-figure 5, in which secondary immunization produced an increased titer of serum agglutinins only for erythrocytes of species of the inoculated cells. The next questions were whether the newly formed hemagglutinins were distinguishable from the natural hemagglutinins and whether the final hemagglutinating activity of antiserum represented combined activities of natural heterophil and induced species-specific agglutinins. Pre- and postimmunization serums



TEXT-FIGURE 5.—Titers of agglutinins for human and rabbit erythrocytes produced in time by guinea pigs receiving intramuscular injections of culture cells mixed with mineral-oil adjuvant. Animals were immunized 5 to 6 weeks earlier with ERK-1, human EE, or rabbit CRE cells as indicated; in groups of 4 these animals were hyperimmunized with 10^6 EE, ERK-1, or CRE cells. Each point represents the titer of induced hemagglutinins in serum from a single animal; time regressions join geometric mean titers.

from animal groups receiving injections of EE, ERK-1, and CRE cells, respectively, were tested by absorption and hemagglutination inhibition by use of whole or disrupted EE, ERK-1, and CRE cells and whole or lysed human or rabbit erythrocytes. Text-figure 6 shows (1) that natural agglutinins were absorbed or inhibited by erythrocytes, but not by cells, of the species of injected cells; (2) cells and erythrocytes of the species of injected cells absorbed or inhibited hemagglutinating activity of antiserums. From these observations, it was concluded that natural and induced hemagglutinins were different in character, that erythrocyte antigens involved in heterophil hemagglutination were not found in or

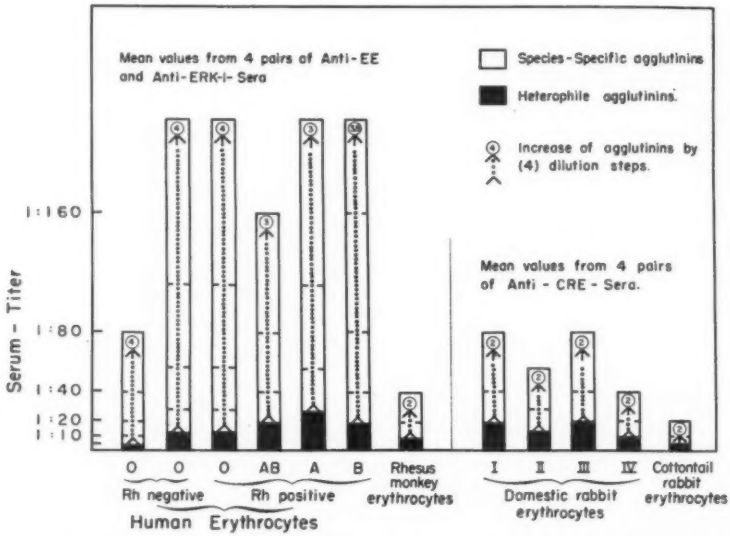


TEXT-FIGURE 6.—Effect of absorption with erythrocytes or culture cells on capacity of guinea-pig serums to agglutinate human or rabbit erythrocytes. Loss of agglutinating activity is expressed on a relative scale by (*a*) assessing serum titers as greatest active dilutions (twofold), (*b*) measuring activity retained after absorption as the percentage ratio of final-to-initial titer reciprocals, and (*c*) expressing activity loss as 100 minus the percentage ratio, thus equating titers of unabsorbed serums. Each bar represents mean values for serums from 4 animals. *Open bars* indicate preimmunization serum groups, and *cross-marked bars*, from *left* to *right*, indicate groups of antiserums to ERK-1, EE, and CRE cells respectively.

on the cultured cells, and that natural heterophil agglutinins were not enhanced by immunization.

Exclusion of Reactions to Blood-Group Antigens

A third question was whether immune hemagglutinins might be produced in response to blood-group antigens in cultured cells since blood-group substances are not confined to man. Antiserums to EE and ERK-1 cells and corresponding preimmunization serums were tested for agglutination of 6 specimens of human erythrocytes of different blood type and erythrocytes of *Macacus rhesus*. Antiserums to CRE cells and corresponding preimmunization serums likewise were tested with 4 specimens of domestic rabbit erythrocytes and with cottontail rabbit erythrocytes. Three of the 4 domestic rabbit specimens differed according to cross match. As shown in text-figure 7, all tests with different erythrocyte samples of the same species as injected cells showed increased content of induced hemagglutinins in antiserums. On an absolute basis, titers differed among serums, apparently because the reactivity of different erythrocyte samples varied for natural as well as induced hemagglutinins. Comparison of the group O, Rh-negative erythrocyte tests indicates that the varying reactivity could not be ascribed to blood groups. Increase in induced antihuman hemagglutinins was revealed, though less strongly, by rhesus monkey as well as human erythrocytes.



TEXT-FIGURE 7.—Agglutinating activity for human and rabbit erythrocytes of guinea-pig serums before and after immunization of animals with culture cells. Each bar represents the mean agglutinating activity of preimmunization (*black*) and postimmunization (*white*) serums from 4 animals receiving injections of EE and ERK-1 (*left*), or CRE (*right*) cells. Increase of induced hemagglutinins, relative to agglutinins in serum of unimmunized animals, is indicated by *dotted arrows*. Agglutinins for human, rabbit, or monkey erythrocyte specimens, as shown, are designated "heterophil" when present in serum before immunization, and "species-specific" when present at indicated levels after immunization with cells of corresponding specificity.

Antigenic Component Common to Rhesus Monkey Erythrocytes and Human Cells

Since antiserums to human EE cells contained hemagglutinins for rhesus monkey erythrocytes, a common antigenic component was indicated. Antiserums to EE, ERK-1, and rhesus monkey kidney cells were compared by cross absorption and hemagglutination-inhibition tests. Results of these tests corresponded; table 1, showing absorption results, indicates that both ERK-1 and human EE culture cells removed rhesus hemagglutinins from antiserums to EE and ERK-1 cells, but failed to remove these hemagglutinins from antiserum to monkey cells. It was concluded that EE and ERK-1 culture cells and monkey erythrocytes, but not monkey culture cells, possess a component in common, as shown by the results of the hemagglutination tests.

DISCUSSION

Immunization of guinea pigs with culture cells and use of antiserum for hemagglutination tests, under suitable conditions, allowed unequivocal

TABLE 1.—Capacity of guinea-pig antiserums to human EE, ERK-1, and monkey kidney cells to agglutinate erythrocytes of different species before and after absorption with EE or ERK-1 cells

Antiserums absorbed with	Erythrocytes agglutinated	EE antiserums			ERK-1 antiserums			Monkey kidney cell antiserums	
		Reciprocal titer in expt.:			Reciprocal titer in expt.:			Reciprocal titer in expt.:	
		I	II	III	I	II	III	I	II
—	Human	320	640	160	640	320	320	<20*	<20
	Rhesus	80	160	80	80	160	80	160	80
	Cynomolgus	<20	<20	<20	<20	<20	<20	80	80
EE cells	Human	20	80	<20	<20	20	40	<20	<20
	Rhesus	<20	40	<20	<20	20	<20	80	80
	Cynomolgus	<20	<20	<20	<20	<20	<20	80	80
ERK-1 cells	Human	<20	40	<20	<20	20	40	<20	<20
	Rhesus	<20	40	<20	<20	20	<20	160	80
	Cynomolgus	<20	<20	<20	<20	<20	<20	80	80

*Lowest dilution tested.

identification of the species origin of human and rabbit cells in early and continuous culture. Interference between immune responses of guinea pigs was negligible, so that cross hyperimmunization and differentiation of primary and secondary responses could be used to confirm the species identity indicated by the simple hemagglutination test. This procedure was sensitive enough to identify the species of rabbit cells contaminating human esophageal epithelial cells in 0.1 percent concentration. Specificity of the hemagglutinin response was not reduced by (*a*) presence in guinea-pig antiserum of induced agglutinins for erythrocytes of species unrelated to the species of injected cells, and (*b*) significant provocation of hemagglutinins by culture mediums or mineral-oil adjuvant.

Although normal guinea-pig serums contained widely reactive heterophil hemagglutinins, specificity of response to immunizing culture cells was readily distinguished by titer increase or by prior absorption of serums with culture cells of the test species. Similar distinction was provided by hemagglutination inhibition. The ready distinction of natural heterophil from specific-induced hemagglutinins indicated that the hemagglutinogens common to cultured cells and erythrocytes were most commonly species specific. This antigenic specificity was emphasized by occurrence of hemagglutinins for rhesus monkey erythrocytes in some guinea-pig antiserums to EE and ERK-1 cells. These cells absorbed the monkey hemagglutinins from their antiserums, but failed to absorb specific monkey hemagglutinins from antiserums to monkey cells. This finding suggests that interference by species-nonspecific antigens, when observed, need not vitiate the hemagglutination test of species identity.

The rhesus monkey hemagglutinogen in the human cells did not appear related to Rh antigens, since it was found for antihuman-cell guinea-pig serums that Rh-negative erythrocytes removed completely the agglutinins for Rh-positive erythrocytes. This hemagglutinogen could not be ascribed to "transformation" or to residence of cells in continuous culture, because it was not active in CRE cells and was active in the untransformed EE strain. Antigens of the OAB and Rh groups did not appear to be involved at all in the activity of antihuman hemagglutinins. While this conclusion appears to discourage investigation of blood-group antigens as a source of differentiation of human cultured cells, it should be emphasized that the species-specific hemagglutination undoubtedly depends on activity of particular antigens of erythrocyte surfaces and possibly of cultured cell surfaces. Different results might be found by use of treated erythrocytes or by study of different cell-antigen immunizing preparations and courses of immunization.

In these experiments the ERK-1 cell strain, reportedly derived from embryonic rabbit kidney, behaved in all respects like a human cell strain. ERK-1 cells contained no trace of rabbit antigens detectable by production of rabbit hemagglutinins in immunized guinea pigs.

The species-specific hemagglutination test may be useful to the study of malignancy *in vitro*, in allowing detection of drastic antigenic changes in cultivated cells beyond species boundaries, if such changes really can occur.

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Immunology of Cultivated Mammalian Cells. II. The Effect of Heterophil Hemagglutinin Content of Serum for Cultivation of Mammalian Cells [1, 2]

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SUMMARY

Pools of "normal" calf serum varied in capacity to agglutinate human and rabbit erythrocytes; some high-titer serums were relatively toxic for growth of human and rabbit cells in continuous culture and for maintenance of human cells in primary culture. Serum toxicity for human and rabbit cells was nonspecifically reduced by absorption with human or rabbit erythrocytes. Lysed erythrocytes of either species added to calf serum improved its quality for cultivation of human and rabbit cells but did not inhibit comparably its capacity to agglutinate erythrocytes. Toxicity of the calf serum notably was decreased in the presence of unheated, compared to inactivated, guinea-pig serum. Neither the ability of calf and guinea-pig serums to agglutinate human and rabbit erythrocytes nor the toxicity of calf serum for cell cultures was reduced appreciably by absorption with human or rabbit culture cells.—J. Nat. Cancer Inst. 24: 1021–1030, 1960.

SERUM FROM different animals, normal or immunized with cultivated mammalian cells, was found to contain naturally occurring agglutinins for erythrocytes of various animal species. Natural hemagglutinins in anticellular serums were distinguished readily by absorption with erythrocytes or culture cells (*1, 2*). It was desirable to know further whether (*a*) heterophil agglutinins could react with culture cells more subtly, and (*b*) natural agglutinins or other antibodies were involved in the varying quality of animal serums for growth or maintenance of mammalian cells. Presence of deleteriously reactive materials in guinea-pig serum, in particular, would complicate tests for cytotoxicity of serums, since common use is made of guinea-pig complement.

This paper is concerned with the relationship between heterophil hemagglutinating capacity of pooled normal calf serum and its effect on growth and maintenance of human and rabbit cells.

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MATERIALS AND METHODS

Cells and cultivation.—Cells used included human esophageal epithelial cells of normal origin in continuous culture (Minn. EE 55-12-1), strain HeLa epithelial cells of human malignant origin, strain ERK-1 epithelial cells reportedly derived from embryonic domestic rabbit kidney, and epithelial cells in continuous culture from normal cottontail rabbit skin (Minn. CRE 57–8–19), as previously described (*2*). Cells of established strains were grown in yeast extract medium (*3*) containing 20 percent serum. Primary cultures of human amnion cells were established from trypsinized cell suspensions in 20 percent human serum in Hanks' balanced salt solution containing 0.5 percent lactalbumin hydrolysate.

Growth inhibition test.—In this study, growth of cultures from dispersed inoculums was graded by the following criteria:

Grade	Culture appearance
0	Cells rounded, detached from glass, and disintegrating.
1	Infrequent single cells attached to glass.
2	Single glass-attached cells in at least 1 microscopic field out of every 3.
3	Single glass-attached cells in every microscopic field.
4	Single glass-attached cells and small colonies of from 3 to 5 cells in each field.
5	Colonies of 3 to 5 cells predominant.
6	Colonies of 20 or more cells with small colonies.
7	Large confluent cell colonies.
8	Cells in light monolayer formation.
9	Cells forming a dense monolayer.

For controls, cultures propagated in serums selected to allow grade 7 to 9 growth in from 3 to 6 days of incubation at 37° C. were used. Cultures were observed by microscope daily until control cultures attained grade 7 to 9 appearance, or as long as control cultures retained grade 7 to 9 appearance. For evaluation of effect on cell-culture growth, test serums in 20 percent concentration were seeded with serial twofold dilutions of trypsinized EE, ERK-1, HeLa, or CRE cells (12,500 to 100,000 cells per 0.5 ml.). Each dilution of cells was inoculated into 3 to 5 tubes of test medium. First-passage human amnion cells were grown in medium containing human serum. Replicate monolayers were washed repeatedly with Hanks' solution and overlaid with 10, 20, or 40 percent serum in maintenance medium (*4*). In growth tests, serums were classified as toxic when cultures exhibited grades relative to grade 7 to 9 control cultures; in maintenance tests, serums were classified as toxic when test cultures with the appearance of initial grades 7 to 9 showed granulation, rounding, and separation of cells, or detachment of cells from glass.

Serum absorption.—Serums were absorbed with erythrocytes as indicated in the description of experiments. Culture cells were prepared and used to absorb serum, as described (*2*).

Hemagglutination and hemagglutination inhibition.—For hemagglutination tests, 0.2 ml. amounts of single serial twofold dilutions of serum inactivated at 56° C. for 30 minutes were dispensed in replicate into cups of vinyl trays; 0.05 ml. of a 0.3 to 0.5 percent erythrocyte suspension

was added to each cup. Phosphate-buffered NaCl solution (0.01 M) at *p*H 7.2 was used as a diluent. The hemagglutinin titer was measured as the serum dilution producing macroscopically visible erythrocyte aggregates. Washed intact or sonically disrupted cells, approximately 10^7 per ml. in buffered NaCl solution, were titrated (0.1 ml. amounts of serial two-fold dilutions) for capacity to inhibit agglutination of erythrocytes by 0.1 ml. amounts of serum dilution containing 2 hemagglutination units in final volume. Inhibition tests with erythrocyte lysates were performed similarly. Tubes were incubated and agitated for 4 to 6 hours at 37° C.

RESULTS

Heterophil Hemagglutinating Capacity of Calf Serum and Cell-Culture Toxicity

In these laboratories, calf serum for cell-culture maintenance for virus research is taken from blood obtained in batches from an abattoir. Calf serum from 13 such pools was titrated for hemagglutinins reactive with human A Rh-positive, human O Rh-negative, rabbit, and sheep erythrocytes. Each serum pool was tested for suitability for growth of EE, ERK-1, HeLa, and CRE cells, and for maintenance of CRE and human amnion cells, as described earlier. Serum classified by either test as toxic with cells of one type was toxic for the other cells, within the limits of classification of toxicity. According to the various grades of effect, serums were more or less toxic or nontoxic for the various cells, but a single serum was not so good by one test and bad by another as to merit both toxic and nontoxic classifications. Results are summarized in table 1. Differences in broad "heterophil" hemagglutinin content of the calf-serum pools were indicated about equally by the rabbit and two types of human erythrocytes but not at all by the sheep erythrocytes. The evidence favored rather than disfavored a correlation between hetero-

TABLE 1.—Agglutination of erythrocytes by normal calf-serum pools* and toxicity for cell cultures

Serum pool	Agglutination titer† for erythrocytes: Human O Rh−	Human A Rh+	Pooled rabbit	Pooled sheep	Toxicity for cell cultures‡
M	128	128	256	<2	+
T1	64	64	32	<2	+
T2	16	32	16	<2	+
F8	64	32	32	<2	+
K5	64	64	32	2	0
8 Pools	<16	<16	<16	<2	0

*Prepared from blood collected at an abattoir.

†Reciprocal of serum dilution producing macroscopically visible agglutination.

‡Serum in 20 percent concentration was used for growth of human EE and HeLa, rabbit CRE, and ERK-1 cells, and for maintenance of human amnion primary cultures. Serum was classified as toxic if maximum growth consisted of single cells and/or colonies of 3 to 5 cells, or if established cultures degenerated, in comparison with control monolayers.

phil hemagglutinating activity of serums and cell-culture toxicity, both quantitatively and qualitatively. However, one calf-serum pool (K5) with considerable hemagglutinating activity was not toxic for any of the cell strains.

Effect of Erythrocyte Absorption on Serum Toxicity

If heterophil hemagglutinating antibodies were involved in the toxicity of the calf-serum pools for cell cultures, serum quality should have been improved by absorption with erythrocytes. Samples of the T1 calf-serum pool were absorbed with washed human A Rh-positive, rabbit, or sheep erythrocytes in 5 percent (v/v) concentration. One serum sample was absorbed with guinea-pig kidney cells in 20 percent (v/v) concentration that was prepared by lightly grinding minced tissue in a mortar, washing the cellular debris three times with buffered saline, and centrifuging the material for 5 minutes at 1500 rpm. Serum samples were absorbed sequentially for at least 1½ hours at 37° C. and/or room temperature and 16 hours at 4° C. The effect of absorption of the calf serum was evaluated by observation of (*a*) reduction of hemagglutinating activity for human and rabbit erythrocytes, (*b*) quality of the serum for growth of EE, HeLa, ERK-1, and CRE cells, and (*c*) quality of the serum for maintenance of human amnion cells when incorporated in medium at 10 and 40 percent levels. Since the toxicity of unabsorbed serum appeared to vary with culture state, effects of absorbed serum on growth were observed with cultures established from different lots of cells. Results are given in table 2. Absorption of serum with human or rabbit erythrocytes, but not sheep erythrocytes or guinea-pig kidney cells, considerably reduced the hemagglutinin content of serum and improved its quality for culture. Human erythrocytes removed specifically the agglutinins for them, and rabbit erythrocytes removed rabbit hemagglutinins. Quality of the serum was nonspecifically improved for growth of all cells by absorption with erythrocytes, more so with human than with rabbit erythrocytes. Within the broad limits of the experiment, absorption with human cells appeared equally effective when performed for shorter times at higher temperature or a longer time at a lower temperature.

Effect of Guinea-Pig Complement on Toxicity of Normal Calf Serum for Cell Cultures

Erythrocytes agglutinated by the normal calf-serum pools used for cell culture were lysed in the presence of guinea-pig complement. If serum elements toxic for cell cultures were hemagglutinins or other antibodies, complement should have enhanced serum toxicity, since complement enhances the action of cytotoxic antibody (*5*). Growth inhibition by the T2 pooled calf serum mixed with uninactivated or inactivated (56° C. for 30 min.) normal guinea-pig serum was tested with EE, HeLa, and ERK-1 cells. The guinea-pig serum was added to culture tubes to a final concentration of 10 percent (v/v). Agglutination titer of the guinea-

TABLE 2.—Effect of absorption with erythrocytes on capacity of calf serum to agglutinate human and rabbit erythrocytes and support growth or maintenance of human or rabbit cell cultures

Serum treatment		Postabsorption hemagglutinin titer for erythrocytes:		Growth of cells with 20 percent serum				Maintenance of human amnion cells with serum	
Absorbing cells	Times absorbed	Human O Rh−	Pooled rabbit	EE	HeLa	ERK-1	CRE	10 percent	40 percent
None	0	64*	32	2, 2, 3, 0, 4†	2	2, 5, 2, 4, 2	0, 0	0‡	0
Human A Rh+	7	4	16	4, 5, 7, 5, 8	5	6, 7, 7, 9, 6	6, 4	+	+
Human A Rh+	2	8	16	7		8, 7		+	+
Rabbit	7	32	<2	2, 4, 3, 2, 6	3	3, 5, 5, 5, 3	5, 2	+	0
Sheep	7	32	16	6		3, 2	0	+	0
Guinea-pig Kidney	2	64	32	5		0	0		

*Reciprocal of serum dilution producing macroscopically visible agglutination.

†Each number resulted from a single experiment in which from 12,500 to 100,000 cells were seeded in test or nontoxic control calf serum into 3 to 5 tubes per inoculum. Results were read when control tubes showed cell monolayers (grade 7-9 growth) as the least degree of growth observed in tubes inoculated with the fewest cells showing observable growth. Each number records the degree of growth according to the density of glass-attached growth centers and the density of cells per growth center qualitatively evaluated.

‡Maintenance of initially healthy monolayers was evaluated by comparison with controls maintained with nontoxic serum; + indicates little difference between tests and controls, while 0 indicates that test cultures contained granular, rounded, separated, or detached cells.

pig serum for human or rabbit erythrocytes was less than one eighth. As shown in table 3, contrary to expectation, uninactivated guinea-pig serum of established complement content improved culture growth markedly in comparison with growth of cultures given heated serum. This result suggested that the toxic serum elements were not complement-dependent antibodies.

TABLE 3.—Effect of heat inactivation on the influence of guinea-pig serum on toxicity of a calf serum for cell cultures

Treatment of guinea-pig serum	Growth of cells in 20 percent calf serum with 10 percent guinea-pig serum		
	EE	HeLa	ERK-1
56° C. for 30 minutes	0, 3*	0	0
None	5, 6	4	4

*Each number represents degree of growth recorded in a single experiment, on a scale ranging from 0 (rounded, disintegrating cells detaching from glass) to 9 (dense cell monolayer). Test cultures were compared with control cultures grown in nontoxic calf serum.

Effect of Lysed Erythrocytes on Toxicity of Normal Calf Serum for Cell Cultures

It was possible that the improving effect of unheated guinea-pig serum on calf serum toxic for cell cultures resulted from addition of labile nutritional factors; if so, the improvement from erythrocyte absorption might be similarly derived. Absorption of serum with erythrocytes was accompanied by some release of hemoglobin, as shown by the pink coloration of absorbed serums. For investigation of this possibility, washed human A Rh+ or rabbit erythrocytes were lysed with distilled water. Lysate was added to a sample of pooled T1 calf serum to produce coloration equal to that resulting from sevenfold absorption with erythrocytes. This degree of coloration was produced by combination of 0.01 ml. of the test lysate preparations with 1.0 ml. of serum. A second serum sample was reinforced with 0.04 ml. of lysate. Table 4 shows relative growth of EE, HeLa, ERK-1, and CRE, and maintenance of human amnion cells in medium containing (*a*) unabsorbed calf serum, (*b*) calf serum absorbed with human erythrocytes, (*c*) calf serum containing 1:100 human-erythrocyte lysate, (*d*) calf serum containing 1:25 human-erythrocyte lysate, (*e*) calf serum absorbed with rabbit erythrocytes, and (*f*) calf serum containing 1:100 of rabbit-erythrocyte lysates. Addition of lysate was no more, and possibly less, effective than erythrocyte absorption in improvement of calf serum for growth or maintenance of cells. Again, the effect appeared nonspecific.

Alternatively, the improving action of addition of erythrocyte lysate on calf serum might have resulted from the blocking effect of heterophil antibodies rather than from nutritional supplementation. Erythrocyte lysates used in the previous experiment were tested for capacity to inhibit agglutination of human erythrocytes by the normal calf-serum pool. Serial dilutions of calf serum (agglutination titer 1/64) were combined, in

TABLE 4.—Effect of supplementation with erythrocyte lysates, in comparison with absorption with erythrocytes, on toxicity* of a calf serum for cell cultures

Serum treatment	Growth of cells with 20 percent serum				Maintenance of human amnion cells with serum		
	EE	HeLa	ERK-1	CRE	10 percent	20 percent	40 percent
None	0, 2, 2	4	4, 5, 2	0	0	0	0
Absorbed 7× with human A Rh+erythrocytes	5, 5, 4	7	9, 7, 5	4	+	+	+
Combined 100 : 1 with human-erythrocyte lysate, A Rh+	3, 4, 4	7	5, 7, 4	4	+	0	0
Combined 25 : 1 with human-erythrocyte lysate	3	7	5		+	0	0
Absorbed 7× with rabbit erythrocytes	4		5	2			
Combined 100 : 1 with rabbit-erythrocyte lysate	3		6	2			

*Capacity of treated or untreated serum to support growth or maintenance of cells in separate experiments was graded (*a*) for growth, on a scale ranging from 0 (rounded, disintegrating cells detaching from glass) to 9 (dense cell monolayer); (*b*) for maintenance, by 0 (degenerative changes in test cultures compared with grade 9 control cultures) or + (similar healthy state of test and control cultures). Control cultures were grown or maintained with nontoxic calf serum.

1.0 ml. amount, with various amounts of human-erythrocyte lysate. Blocking capacity of lysates appeared proportional to the lysate-serum ratio. On this basis, 0.01 ml. of lysate combined with 1.0 ml. of calf serum (a combination previously shown to improve calf serum for cell culture) could be considered capable of inhibiting no more than 9 to 17 percent of the heterophil h magglutinating capacity of the serum. In a similar test, human-erythrocyte lysate did not inhibit agglutination of rabbit erythrocytes. Specificity of the hemagglutinin-blocking activity of human-erythrocyte lysate contrasted with the nonspecific capacity of lysate to improve calf serum for growth of human and rabbit cells.

Effect of Absorption With Culture Cells on Heterophil Hemagglutinating Capacity and Toxicity of Normal Calf Serum for Cell Cultures

As another approach to the relation of heterophil serum hemagglutinins to toxicity of serum for cell-culture growth and maintenance, serum of the calf T1 pool, or guinea-pig serum with heterophil hemagglutinins to high titer, was absorbed with EE, ERK-1, or CRE cells. Absorption of 1.0 ml. of serum diluted one half, with about 10^6 cells, did not reduce the capacity of calf or guinea-pig serum to agglutinate human or rabbit erythrocytes. Second serum samples were absorbed with 10^7 cells per ml. of serum diluted one half. Heterophil hemagglutination titers of both the normal calf and guinea-pig serum were unaltered by absorption with EE or CRE cells. Absorption with ERK-1 cells reduced the capacity of calf serum but not guinea-pig serum to agglutinate human and rabbit erythrocytes to a small extent.

Hemagglutination-inhibition tests were carried out with the calf and guinea-pig serums and with EE and ERK-1 cells. Whole EE cells did

TABLE 5.—Effect of absorption with culture cells or erythrocytes on capacity of calf serum to agglutinate human or rabbit erythrocytes and support growth of human and rabbit cell cultures

Treatment of calf serum	Agglutination titer* for erythrocytes		Growth of cells with 20 percent serum		
	Human O Rh−	Rabbit	EE	ERK-1	CRE
None	64	32	3†	0	0
Absorbed with EE cells	64	32	2	2	0
Absorbed with ERK-1 cells	16	16	3	2	0
Absorbed with human A Rh+ erythrocytes	8	32	7	5	5
Absorbed with rabbit erythrocytes	64	8	5	4	3
Supplemented with lysed human A Rh+ erythrocytes	64	32	7	4	5
Supplemented with lysed rabbit erythrocytes	64	32	4	3	2

*Reciprocal of serum dilution producing macroscopically visible agglutination.

†Degree of growth recorded on a scale ranging from 0 (rounded, disintegrating cells detaching from glass) to 9 (cell monolayer). Growth was evaluated when control cultures in nontoxic calf serum reached grade 7 to 9 state.

not inhibit human or rabbit erythrocyte agglutination by calf or guinea-pig serum. With whole or sonically disrupted ERK-1 cells, only slight and unstable blocking of heterophil hemagglutination by calf but not guinea-pig serum was observed.

The calf-serum samples absorbed with EE and ERK-1 cells were tested for their effect on growth of EE, ERK-1, and CRE cells, in comparison with other samples of calf serum absorbed with human or rabbit erythrocytes or supplemented with lysed human or rabbit erythrocytes. The results indicated in table 5 verified the capacity of human and rabbit erythrocytes to improve the quality of calf serum for culture growth. Serum absorbed with culture cells, however, was no better than untreated serum. The inability of culture cells to affect toxicity of the normal calf serum indicated that the cells did not contain antigenic components or other materials capable of absorbing the toxic serum factor(s). Inability of cells to absorb or block heterophil hemagglutinins indicated similar lack of relation of cell components to erythrocyte hemagglutinin receptors.

DISCUSSION

The question asked in these experiments was whether natural heterophil hemagglutinins in serum could affect the growth or maintenance of culture cells of corresponding species. Calf serum that agglutinated human and rabbit erythrocytes was used for growth of human and rabbit cells in continuous culture and for maintenance of human cells in primary culture. Relation between heterophil hemagglutinin content of the serum and toxicity for cell cultures was indicated by (*a*) some qualitative correlation between hemagglutinin titer and toxicity and (*b*) improvement of serum quality for culture by absorption with erythrocytes. The following results contraindicated such a relation: (*a*) One serum pool was found without any toxicity for cell growth despite high titers of heterophil hemagglutinins; (*b*) erythrocyte absorption improved toxic calf serum nonspecifically, while heterophil hemagglutinins were removed specifically; (*c*) addition of fresh guinea-pig complement enhanced cell growth rather than exacerbated serum toxicity; (*d*) addition of erythrocyte lysate improved serum quality for culture at concentrations incapable of blocking hemagglutinins effectively; (*e*) culture cells were unable to absorb or inhibit hemagglutinins present in calf or guinea-pig serums or to decrease toxicity of calf serum by absorption. Although heavy concentrations of ERK-1 cells reduced heterophil human and rabbit hemagglutinin content of calf serum slightly, the course of hemagglutination inhibition with disrupted ERK-1 cells suggested only nonspecific reversible binding of hemagglutinins by cell substances.

These results apparently excluded action of conventional antibody as a basis for toxicity of a heterophil hemagglutinating serum for cell cultures. Exclusion of combining antibody agrees with the previously reported (*1*, *2*) susceptibility of natural serum hemagglutinins to absorption by

erythrocytes but not cells, in contrast to the susceptibility of specifically induced hemagglutinins to absorption by cells as well as erythrocytes. The observed differential effect of heated and unheated guinea-pig serum on serum toxicity, independent of conventional complement function, is a factor for consideration in the performance of tests for cytotoxic serum antibody. Heated guinea-pig serum commonly is used as a complement control.

Although this investigation was intended to assess the possible relationship of heterophil hemagglutinins to cultured cells of homologous species, and indicated no specific relationship, the nonspecific (in the usual sense) effects are worthy of note. It may be advisable to absorb cytotoxic serums used in growth-inhibition tests with heterologous erythrocytes to reduce the content of heterophil substances without reducing the content of species-specific antibody. Where heterologous serums are used for cell culture, erythrocyte absorption may be a useful measure to improve serum quality.

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A Highly Inbred Line of Wistar Rats Yielding Spontaneous Mammo-somatotropic Pituitary and Other Tumors [1, 2]

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SUMMARY

Spontaneous neoplasms of female rats of a highly inbred Wistar line are described. All neoplasms tested were found to be transplantable within the strain; many were hormone-dependent. Pituitary tumors were the most common neoplasm occurring in over 27 percent of rats surviving beyond 17 months of age. These "chromophobe adenomas," like those induced by estrogens, were functional, having marked mammosomatotropic effects, and were variously responsive to estrogens. The secondary changes were more marked in rats with grafted than with spontaneous tumors. The almost equally common mammary fibroadenomas proved transplantable to all rats conditioned with grafted mammosomatotropic tumors but to only a few rats without them. Thus, these tumors appear to be autonomous but responsive to the mammotropic hormone(s) of the anterior pituitary. Their poor transplantability was due not to heterozygocity of the Wistar strain but to hormone dependence. For example, whereas transplantation succeeded in only 2 of 11 normal females (3 strains) after about a year of latency, all 3 mammary-tumor grafts in mammotropic-tumor-bearing animals took in all 13 rats after a mean latent period of 70 to 216 days. Secondary changes in the host suggested that the spontaneous adrenocortical tumor observed secreted corticoids. Three spontaneous leukemias and one malignant lymphoma tested proved transplantable to all adult animals of the strain. The usefulness of this strain in studies of the genesis and control of mammary tumors, leukemias, and some endocrine neoplasia is discussed.—J. Nat. Cancer Inst. 24: 1031–1055, 1960.

ALTHOUGH THERE have been many reports in the literature on the incidence of spontaneous tumors in various strains of rats (*1–7*) this species has not been used to full advantage in cancer research, in part because of poor transplantability of tumors due to the heterozygocity of the strain and in part because of inadequate knowledge of the spectrum of tumors and their character in the available inbred strains of rats.

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In the highly inbred subline of Wistar rats (W/Fu) developed in our laboratories, a high incidence of spontaneous pituitary and mammary tumors and other neoplasms, including leukemias, has been noted. All spontaneous neoplasms tested proved 100 percent transplantable within the strain. The pituitary and adrenal tumors proved to be functional and hormone-responsive. It is the purpose of this communication to describe the incidence and character of the neoplasms noted in this strain and point out its usefulness in the study of diverse problems of cancer research for which the rat is more suitable than the mouse. The rat strains will be designated W/Fu (Wistar/Furth) and F/Fu (Fischer/Furth), following accepted terminology of mouse geneticists.

MATERIALS AND METHODS

Origin of the W/Fu line.—Approximately 13 years ago numerous malignant lymphomas were observed in a then partly inbred line of Wistar rats. One of us (J.F.) began inbreeding this strain by consecutive *brother* × *sister matings*, hoping to develop a high-leukemia rat strain. This aim has not been achieved; in spite of continued inbreeding, leukemias and lymphomas occur in this strain only in small numbers. The strain was used extensively in Oak Ridge (1949–53) for attempted induction of pituitary tumors by I^{131} (*8*), and for studies of radiation effects, notably cataract induction (*9*). In Boston (1954–59), it was used for studies of transplantable adrenal (*10*), pituitary, and mammary tumors, and leukemias (*11*).

Breeding.—Animals were selected for mating from large litters. In most cases breeding was continued until senescent infertility. The females had thus borne 1 to 6 litters. All females of the pedigreed lines were kept until natural death if not killed for study of neoplasms. Male rats, for the most part, were not kept until natural death.

Maintenance.—The diet consisted of Purina laboratory chow, supplemented once weekly with lettuce and carrots, and tap water *ad libitum*. During pregnancy and lactation, milk and bread were also given daily. To control infectious diseases, veterinary terramycin was administered in drinking water when indicated. Animals were housed in coarse wire-mesh suspended cages until advanced pregnancy. Before parturition females were moved to individual whelping pens with wood shavings and sawdust as nesting material, where they remained throughout lactation. Litters were weaned 3 to 4 weeks after birth.

Histologic examination.—All pedigreed animals were autopsied and histological sections prepared as needed. Special attention was given to the endocrines and their target organs and to hematopoietic tissue. Tissues were fixed in 5 percent Zenker-formol solution and stained with hematoxylin and eosin. The pituitary gland was also stained with Gude's modification of Mallory-Martin trichrome stain (*12*). Blood smears were stained with Wright and Giemsa stains.

Transplantation.—Tumor and leukemia grafts were made on rats of the W/Fu strain or F_1 hybrids of the W/Fu and Fischer (F/Fu) strains. Tumors

were minced in saline or Eagle's basic tissue-culture medium, to which penicillin and streptomycin were added, and injected intramuscularly. In recent experiments, solid tumors were transplanted subcutaneously in thin strips or in small pieces intramuscularly, thereby shortening the latent period. Leukemias were usually transmitted by intraperitoneal injection of spleen-cell suspension.

Special treatment.—Gonadectomies and adrenalectomies were performed by standard surgical procedures under ether anesthesia. Hypothyroidism was induced with a low-iodine diet [5] mixed with 0.02 percent desiccated thyroid powder given in addition to 0.02 percent propylthiouracil (PTU) [6] in the drinking water. Adrenalectomized animals were given 2.5 mg. deoxycorticosterone trimethylacetate [7] subcutaneously at 3- to 4-week intervals and 1.0 percent salt water *ad libitum*. For estrogenization, pellets containing 0.5 or 1.0 mg. of diethylstilbestrol (DES), fused with 9.5 or 19 mg. cholesterol, respectively, were implanted subcutaneously and were replaced at about 6-month intervals. To induce high levels of mammotropic hormones, autonomous mammotropic pituitary tumors (MtT) were grafted subcutaneously in the same hosts (*13*, *14*). When the grafted MtT exceeded approximately 2 cm. in diameter they were surgically resected.

RESULTS

Frequency and types of major diseases.—The tumor types, their frequency, and their time of occurrence in female W/Fu rats are summarized in table 1. In a total of 83 pedigreed rats autopsied in Boston (1955–59)

TABLE 1.—Spontaneous-tumor incidence in female W/Fu rats

Type or organ	Tumor		Age (*months*)		Number in group*
	Number	Percent	Extreme	Median	
Pituitary	20	27.3	17–33	23	73
Mammary	17	21.2	12–31	20	80
Adrenal	2	2.5	14, 30		78
Uterine	2	2.4	10, 20½		83
Leukemias (generalized)	6	9.1	19–30½		66
Lymphomas (malignant)	2	7.1	24, 30		28
Lipoma	1	2.6	23		39
Unclassified	3	3.6	10–22		83

Total number of rats over 10 months of age	83
Total number of tumor-bearing animals	46 (55.4%)
Total number of tumors	53
Animals bearing 2 tumors	7 (15.2%)

*Total number of rats of same age or older than the youngest tumor-bearing animal.

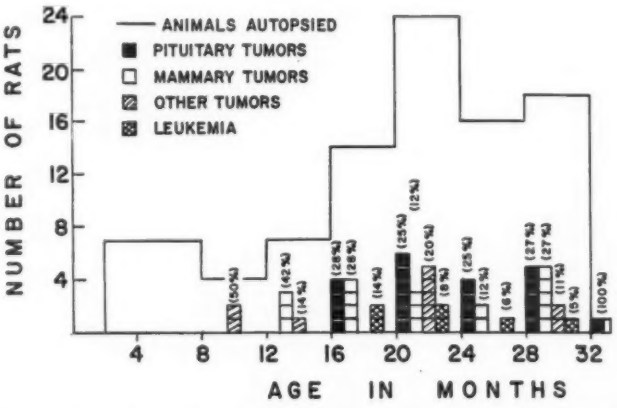
[5] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[6] Generously supplied by Dr. S. M. Hardy, Lederle Laboratories Division, American Cyanamid Corporation, Pearl River, N.Y.

[7] Generously supplied by Dr. Robert Gaunt and Dr. A. A. Renzi, Microbiology Laboratories, Ciba Pharmaceutical Co., Summit, N.J.

at 10 to 33 months of age, 53 tumors occurred in 46 animals (55.4%). Seven rats had 2 tumors. Among the 20 spontaneous pituitary tumor-bearing animals, 3 also had mammary fibroadenomas, 1 had leukemia, 1 a leiomyoma of the uterus, and 1 a presacral lipoma. One of the fibroadenoma-bearing animals also had a lymphoma.

The number of neoplasms, divided into four major categories, is plotted in 4-month units (text-fig. 1). The incidence of various tumor types is also plotted as a percentage of the total number of animals autopsied in each group.



TEXT-FIGURE 1.—Incidence of spontaneous neoplasms in female W/Fu rats.

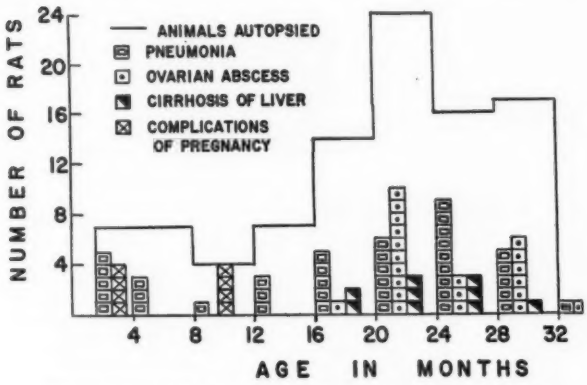
The text-figure shows that both pituitary tumors and leukemias occur in animals over 16 months of age. Mammary tumors were relatively evenly distributed. Mammary tumors began to appear at 13 months of age, pituitary tumors at 17 months, and leukemias at 18 months. In earlier series, not tabulated because of lack of completeness of the data, leukemias were seen in younger rats, but pituitary tumors were not seen in animals younger than 17 months.

Text-figure 2 illustrates the incidence of non-neoplastic diseases in 98 female rats. Pneumonia was the most common cause of death. Ovarian abscesses and cirrhosis of the liver were found mostly in animals older than 19 months of age. Fatal complications of pregnancy occurred among the mothers less than 10 months of age.

Tumors of Pituitary Gland

Tumors of the anterior lobe of the pituitary gland, the largest single group of spontaneous neoplasms, accounted for nearly 38 percent of all tumors and were found in 27 percent of all female rats autopsied when older than 17 months. All were adenomas.

Anatomic features.—These tumors were roughly spherical, measuring approximately 5 to 10 mm. across. They were circumscribed, protruded



TEXT-FIGURE 2.—Incidence of non-neoplastic diseases.

from the sella (fig. 1), and compressed the brain. They were soft, friable, and usually extremely congested.

Unlike those in radiation-induced mouse pituitary tumors (*17*), these tumor cells did not invade the bone marrow of the sella and adjacent brain. They were roughly polyhedral and but slightly variable in size. The cytoplasm was moderately abundant, containing fine, pale-pink-staining granules in sections stained with hematoxylin and eosin. The nuclei were large, round or oval, moderately chromatic, and usually centrally located. Occasional giant tumor cells with markedly pleomorphic and hyperchromatic nuclei were found (fig. 2). Mitotic figures were few. The cells appeared in clusters interspersed with markedly dilated capillaries and small irregular strips of compressed normal pituitary components. Hemosiderin-laden macrophages were occasionally present as well as areas of fresh hemorrhage.

In addition to tumor cells with lightly stained fine acidophilic granules, some containing very large acidophilic granules were also noted, as described by Wolfe and Wright (*18*). These granules stained pink to dark red with Gude's modified trichrome. The cells of the grafted tumor (fig. 4) were similar to those of the spontaneous tumor (fig. 2).

Physiologic features.—Gross inspection disclosed slight to moderate stimulation of the mammary glands, predominantly of the ductal type in animals bearing primary pituitary tumors. The mammary-gland stimulation was more marked in rats with large grafted pituitary tumors (figs. 5 and 6) than with primary tumors (fig. 3). Microscopically, both acini and ductal spaces had increased in number and were distended with amorphous acidophilic material. No atypicality or anaplastic changes were noted in either the acinar or ductal epithelium. Other endocrine organs showed no noteworthy changes.

Transplantation.—In order to characterize the functional capacity of these neoplasms, 5 were transplanted in normal and variously conditioned hosts (table 2). All proved to be autonomous but responsive to hormones. The small number of recipients in transplantation study was due to

TABLE 2.—Transplantability and latency of spontaneous pituitary tumors in variously conditioned hosts*

Tumor strain	Passage	♀	♀+DES†	Adrex. ♀	♀+PTU	♂	♂	♂+DES‡	♂+PTU
W2	Orig.	*2/2(117)*	3/4(185)	0/3	0/4				
	Ia,b	1/5(296)				0/5	0/3	*2/6(232)*	
	IIa§						4/5(117)	*6/6(84)*	
	IIb¶	*6/6(67)*				0/4		5/6(70)	
	IIc§						0/5	*5/5(20)*	
W3	Orig.					2/3(316)		*1/3(97)*	1/5(365)
	Ia					*2/2(301)*		0/2	
W4	Orig.	*4/6(263)*	0/6						
W5	Orig.	1/3(442)	*3/4(262)*						
W6	Orig.					1/2(226)		*4/4(113)*	
Total		14/22(64%)	6/14(43%)	0/3	0/4	5/16(31%)	4/13(31%)	23/32(72%)	1/5(20%)

*The symbols ♀ and ♂ indicate castrate females and males, respectively.

The first 2 number in all columns indicate number of takes over number of rats injected. The numbers in parentheses show the mean latent period, in days. For each passage, the group with the shortest latent period is shown in *italics*.

†DES, 0.5 mg., in subcutaneous pellet.

‡DES in subcutaneous pellet, 1.0 mg. in subpassage of tumor strain W2, 0.5 mg. in others.

§Passage IIc made 5.7 days later in opposite thigh of same animals as passage IIa.

¶Most animals were alive when the data were summarized.

scarcity of these rats. The data, though not extensive, indicate that growth of these tumors is strongly influenced by estrogens.

The percentage of takes was highest in castrated DES-treated males (averaging 72%) and in normal females (averaging 64%). The tumors took in 31 percent of normal males, but, remarkably, castration did not increase the success of transplantation.

In general, the latent periods paralleled the success of transplantation. The original grafts of strains W2 and W4 grew poorly in DES-treated rats. In subpassage Ia and Ib, strain W2 grew only in females or DES-treated castrated males. On further subpassage, the latent period was markedly decreased, but responsiveness to estrogen persisted.

Rats with MtT grafts of all 5 strains showed marked mammo-somatotropic effects. The mammary glands of 18 rats bearing tumors of the original transplant generation, measuring 5 to 35 mm. in diameter, were examined microscopically. The proliferative changes were most pronounced in intact females, but large milk cysts were absent in these. In intact males, estrogen-treated castrated males, and estrogen-treated females, proliferative changes were slightly less marked, and milk cysts, measuring 5 to 15 mm. in diameter, were common.

There was a concurrent acceleration of the body growth of tumor hosts with disproportionate enlargement of parenchymatous organs, such as liver, spleen, and kidneys (text-fig. 3, fig. 6), frequently resulting in gigantism. The secondary changes in these tumor hosts parallel those noted in rats bearing estrogen-induced MtT (*14*, *19*). The *liver* enlargement was often coupled with fatty degeneration and the kidney enlargement with nephrosis. These effects occurred in rough proportion to the tumor size and were conspicuous when the tumors were very large.

The *adrenals* of tumor hosts were enlarged up to four times the normal size. The adrenal cortices were thickened and light brownish-yellow. Microscopically, the cortical cells appeared to be somewhat enlarged, but the increased size seemed mostly due to fatty infiltration in the cytoplasm

TEXT-FIGURE 3.—Somatotropic effect of grafted spontaneous pituitary tumors.

The thymus of these animals was often of normal size, indicating absence of a hypercorticoid state.

The *ovaries* were of normal size and often had retained corpora lutea. The uteri were not enlarged. The *testes* and seminal vesicles of the intact males with tumor grafts showed no significant change. Thyroid glands were normal.

These tumors resembled those induced by estrogen (DES) (fig. 7), which caused similar mammary-gland stimulation (fig. 8).

Tumors of Mammary Gland

Mammary tumors were second in frequency to pituitary tumors, comprising 32 percent of all neoplasms. All tumors here recorded were fibroadenomas, although, earlier, a few carcinomas were also noted in the strain, but not sarcomas.

Anatomic features.—The tumors ranged up to 8 cm. in maximum diameter at the time the animals died or were killed. They were nodular, lobulated, well encapsulated, and "rubbery"-firm. Microscopically, they varied in cellularity, ranging from masses of almost solid connective tissue to abundant clusters of acinar and ductal elements surrounded by a scanty fibrous stroma. Most of them were fibroadenomas and a few could be considered adenomas. All were benign, though they tended to grow rapidly.

Transplantation studies.—Four of these tumors were grafted subcutaneously or intramuscularly. The latent period of grafted tumors was very long and the percentage of takes was low in untreated hosts.

The hormonal responsiveness and transplantability of these tumors are indicated in table 3. Although the data are not extensive, it is of special interest to note that grafts of 3 tumors grew in all animals that also bore mammotropic pituitary tumors, *i.e.*, in which MtH levels were greatly increased, and poorly or not at all in other hosts. It follows that poor transplantability of these fibroadenomas is due not to genetic heterozygocity but to lack of conditioning hormonal stimulation. Note also the marked reduction of latent period in the mammotrope-stimulated rats.

Adrenal Tumors

Both tumors observed were cortical adenomas. The younger of the 2 animals was extremely obese. It had a unilateral tumor measuring approximately 2 cm. in diameter; the contralateral adrenal was atrophic (fig. 10). The tumor was well encapsulated and yellow with a necrotic center. It was composed of relatively uniform cortical cells without particular architectural arrangement (fig. 9). Some cells had occasional lipide vacuoles in the cytoplasm; extensive fatty infiltration was noted in the stroma. The thymus was completely involuted. There was some osteoporosis. The uterine mucosa was atrophic and fibrous, and the ovaries bore small corpora lutea. The mammary glands were fatty and

TABLE 3.—Effect of hormonal conditioning of hosts on transplantability and latent period of grafted fibroadenomas of W/Fu rats*

Tumor strain and passage	Site	Sex and treatment of host					
		♀	♀ + DES	♂	♂ + DES	♂ + MtT	♂
W2, original	i.m.	1/7(373)	0/6			7/7(216)†	
W3, original	s.c.			0/5	1/4(222)	*3/3(70)*	
First subpassage	s.c.	1/2(362)	*3/4(345)*				
W4, original	s.c.			2/4(198)	4/4(207)	*3/3(70)*	
First subpassage	s.c.	0/2	*1/4(362)*				
W5, original	i.m.			0/1	0/3		*1/2(396)*
Total		2/11(18%)	4/14(28%)	2/10(20%)	5/11(45%)	13/13(100%)	½(50%)

*The symbols ♀ and ♂ indicate castrate females and males, respectively.

The first 2 numbers in all columns indicate number of takes over number of rats injected. The numbers in parentheses show the mean latent period, in days. For each passage, the group with the shortest latent period is shown in *italics*.

†Also received single pellet of 0.5 mg. DES.

the glandular elements were scanty. The pituitary was not remarkable. These findings bear some resemblance to Cushing's syndrome.

The functional character of the other adrenal tumor seemed somewhat different. It also was a unilateral cortical adenoma measuring approximately 1.5 cm. in average diameter. The opposite adrenal was atrophic and the thymus was involuted as in the first case. Microscopically, the tumor was composed of uniform cortical cells arranged in cords and nests along dilated capillaries. The cytoplasm was relatively dense and acidophilic. Many mitotic figures and occasional anaplastic areas were found. Sections of pituitary showed nodular hyperplasia of eosinophils, some of which contained small cytoplasmic vacuoles. In spite of the animal's advanced age, the endometrium and mammary glands were hyperplastic. This suggests production of an estrogenic hormone by the tumor. Confirmatory evidence of atrophic ovaries was lacking, however, due to large bilateral ovarian abscesses. These tumors were discovered in dead animals and no grafts were made.

Leukemias, Generalized

The 6 leukemias here reviewed constitute approximately 9 percent of all tumors. In addition, several presumably spontaneous leukemias have been observed in experimental W/Fu animals. One developed in a rat that bore a grafted adrenal tumor and another occurred in a year-old rat that had received a graft of a spontaneous pituitary tumor. One of the 7 animals listed in table 1 had leukemia in addition to a pituitary tumor. Two leukemias of this series were derived from the offspring of a single male that also had a leukemia. Extensive attempts to inbreed his offspring for leukemia were unsuccessful.

Anatomic features.—The white-cell counts of the leukemic animals ranged from 100,000 to 500,000. Fifty to 90 percent of these were blasts (figs. 11 and 12); the rest were mainly atypical or immature lymphocytes. The chromatin pattern was coarse and structureless. The cytoplasm of these leukemic cells was moderately abundant and slightly to moderately basophilic; that of another strain contained many fine azurophilic granules (figs. 13 and 14).

Opinions varied as to the type of these cells. Leukemia was associated with progressive anemia. Terminally, the nucleated-red-cell counts were usually high. There was always marked splenomegaly with moderate hepatomegaly with or without generalized lymphadenopathy. Liver and spleen were usually flooded with the leukemic cells, and bone marrow was extensively infiltrated by them.

Transplantation studies.—Four leukemias tested proved to be 100 percent transplantable in the W/Fu strain, regardless of sex or age of the hosts, and at present 3 of these are carried by Maddock.[8] As reported by Maddock (*11*), established leukemias follow in acute course, killing most rats within 3 weeks.

[8] Personal communication.

Lymphomas, Malignant

The two lymphomas had generalized lymphatic involvement at autopsy. The sections failed to disclose leukemic blood involvement. Enlargement of the mesenteric lymph nodes was most conspicuous. When one lymphoma was grafted, it produced a local tumor without blood involvement. On this basis these two cases are discussed separately from leukemia. In the spontaneous cases there was marked generalized enlargement of lymph nodes, involving superficial and deep-seated nodes, slight hepatomegaly, and slight to moderate splenomegaly. Some infiltrated nodes attained a diameter of 2.5 cm. The central areas of the larger nodes were often hemorrhagic. The microscopic picture of this lymphoma showed replacement of a node by large cells, either lymphoblasts or reticulum cells or both (fig. 15). The bone marrow was not distinctly involved and blood smears showed only a few atypical lymphoid cells.

Transplantation studies.—One lymphoma tested was 100 percent transplantable in W/Fu rats of either sex. The latent period in the original passage ranged from 2½ to 4 months and in the second subpassage was less than 1½ months.

Miscellaneous Tumors

A tumor of the *uterus* located in the left horn was firm and nodular and measured approximately 1.0 cm. in diameter. The cut surface was pearly white and displayed a whorl-like pattern. Microscopically, it was a *leiomyoma* composed of bundles of smooth muscle fibers and fibrous connective tissue.

Two of the 3 tumors of unknown origin were carcinomas found in 15- and 22-month-old female rats. The abdominal cavity was filled with small tumor nodules and both ovaries were involved. The site of origin of these tumors, however, remained undetermined. Both were composed of small, uniform cells containing hyperchromatic nuclei occurring in sheets and cords. Mitotic figures were numerous, with incompletely formed acini.

The third tumor was found in a 10-month-old animal, the youngest in this series. It involved the right ovary and distal uterine horn, and measured $4 \times 4 \times 3$ cm. It was dark red, spongy, and succulent. The tumor was composed of numerous congested capillary spaces with marked endothelial hyperplasia but no frank anaplasia of the endothelial cells. The tentative diagnosis was ovarian hemangioma.

The *lipoma* found in the presacral region measured $1.5 \times 1.0 \times 1.0$ cm. It was moderately soft, well encapsulated, and pale yellow on the cut surface. Microscopically, the tumor was composed of loosely knit immature fat cells. Areas of myxomatous degeneration were noted. There were no mitotic figures or anaplasia.

DISCUSSION

Tumors of the pituitary gland.—Since Fisher first described 2 spontaneous pituitary tumors in albino rats (*20*), it has become known that "chromo-

phobe adenomas" are common in old rats (*21–24*). The largest single series was reported by Saxton *et al.* (*1*), who listed 92 chromophobe adenomas in 236 spontaneous tumors (approximately 38%) in the Yale strain of Osborne-Mendel rats. The incidence in our series is similar and higher than that reported for other Wistar lines (*2, 3, 22*).

It is generally supposed that chromophobe adenomas are nonfunctional, but studies in our laboratories indicate that these are composed of a heterogeneous group of functional, poorly granulated cells. The spontaneous pituitary tumors of W/Fu rats are morphologically and functionally nearly identical to those induced by chronic administration of estrogens *(13, 25–29)*. None of the tumors had adrenotropic, gonadotropic, or thyrotropic effects, as suggested by Saxton *et al.* (*24*) on the basis of bioassays of spontaneous tumors in rats.

All spontaneous pituitary tumors transplanted by us were autonomous in the original passage, as were similar tumors induced in mice by radiation (*30*). In contrast most estrogen-induced MtT were fully hormone-dependent in the original passage. These data support the concept that the mammo-somatotrope (Mt) is a distinct pituitary-cell type with mammary-gland-stimulating (*31–33*) and growth-promoting actions.

The pathogenesis of these tumors is unknown. It seems probable that their high incidence in old females is related to repeated stimulation by the heightened estrogen levels of estrus, pregnancy, and lactation.

Pituitary tumors in old males were also described by others (*1, 21, 24*), but little is known about their fundamental characteristics.

Bioassays of hormones.—The nature of hormone(s) secreted by functional MtT has not been fully clarified. Prolactin activity is affirmed by Meyer and Clifton (*15*) and Bates *et al.* (*16*), using the pigeon-crop assay. Lyons [9] assayed MtT and found marked growth-promoting but only slight mammary-gland-stimulating and no luteotropic activity. Lyons doubts that the mammary-gland stimulation is due to prolactin secreted by these tumors.

Hormone levels in a gland or tumor need not correlate with the amount of hormone released by that organ or tumor; *i.e.*, hormones may be stored, giving rise to high levels in the gland and low levels in the blood. Conversely, the rate of hormone release may approach the rate of synthesis, which would give rise to low levels in the gland and high circulating levels. A conventional assay may not reflect the extraordinary stimulation seen in a host bearing an MtT and steadily discharging hormones over a long period.

Mammary tumors.—The responsiveness of fibroadenomas to estrogens has been well established by others (*34, 35*). Our data suggest that the stimulating effect of estrogens is mediated by Mt of the pituitary, *i.e.*, estrogen stimulates the Mt to release MtH, which in turn stimulates growth of the mammary gland (*13*). Growth of these grafted tumors was poor in unconditioned hosts but excellent in rats bearing MtT. MtT not

[9] The tentative conclusions of Dr. William R. Lyons are cited with his kind permission.

only increased the percentage of tumor takes, but also hastened proliferation of neoplastic epithelial cells. Failure of growth of tumor grafts in all rats not bearing MtT is not due to heterozygocity of the strain but to their dependence on MtT. MtT does not lift the barriers of histocompatibility. That others were more successful in transplanting fibroadenomas may have been due to use of larger and more glandular tumor fragments or fragments with greater autonomy.

Tumors of the adrenals.—The transplantable, highly functional adrenal tumors studied by Cohen *et al.* (*10*) were derived from radiothyroidectomized W/Fu rats not included in the present series, even though we believe that these tumors were also spontaneous. Glucocorticoid and mineralocorticoid activities of these tumors were proved to be due to the corticosterone they secreted. The autopsy findings of the present series suggest that one tumor secreted gonadal in addition to corticoid hormones. Recently, in Osborne-Mendel rats, Snell and Stewart (*36*) reported a transplantable spontaneous adrenocortical carcinoma with functional characteristics similar to those described by Cohen *et al.* Sex hormone-producing cortical tumors are reported in old gonadectomized mice of certain strains (*37*). Corticoid-secreting adrenal tumors are suitable for an assay of corticotropin (ACTH) *in vitro* (*38*).

Leukemias and lymphomas.—The low incidence of leukemias in rats and the lack of well inbred strains has limited the use of this species in leukemia research.

Although a few cases of lymphosarcoma had been seen in laboratory and wild rats earlier, spontaneous leukemia was not reported until 1936, when Wilens and Sproul described 12 cases in Osborne-Mendel rats. Eleven were myeloid, including 4 of the chloro-type; 1 was lymphoid. Rask-Nielsen described a transplantable aleukemic myeloblastoma of the mesentery in one of 30 old rats examined [for historical references *cf.* (*39*)]. Oberling *et al.* (*40*) found 6 lymphoid and 3 myeloid leukemias in 6,000 rats. Shay *et al.* (*41*) noted 2 myeloid and 6 lymphoid leukemias in random-bred Wistar rats treated with methylcholanthrene and extensively studied transplantable leukemias derived from them (*42*, *43*). Dunning *et al.* (*44*) have developed, in the Fischer strain, a transplantable leukemia that originated in a rat bearing grafted mammary tumor.

The leukemias in W/Fu rats are morphologically similar to those described by Oberling *et al.* (*40*), who emphasized the difficulty in classifying them. After extensive tests, Maddock (*11*) reported that W/Fu leukemias were ". . . relatively resistant to the common tumor inhibitory agents tried, and in this respect they more closely resemble the human disease than other transplantable rodent leukemias."

The terms "lymphoid leukemia," "lymphoma," and "lymphosarcoma" are often used as if they were entirely different entities. In our tabulation, the distinction was made arbitrarily between the "systemic" disease (leukemia), with high circulating white cell counts and no local tumors, and localized tumors (lymphoma or lymphosarcoma) without blood involvement. In most cases, the latter seemed to have originated in the

mesenteric lymph nodes. The pathogenesis of these two types of "hemoblastosis" may be different.

Usefulness of the W/Fu strain.—Limited availability of highly inbred strains of rats has hitherto hampered research with rat tumors, although some investigators have used partially inbred rats very extensively, *e.g.*, Huggins *et al.* (*34, 35*) for research in mammary tumors and Haddow and associates (*45*) for cancer chemotherapy. Increased availability of highly inbred rat strains will much enhance the usefulness of this species.

a) The mouse is not well suited for studies of human mammary-tumor problems because nearly all mouse mammary tumors are fully autonomous. In rats, on the contrary, mammary tumors are highly responsive, as first emphasized by Huggins *et al.* (*34*). With noninbred strains, the percentage of tumor takes varies greatly, and studies are limited to the animal bearing primary tumors. With the well inbred W/Fu strain, grafts of the first transplant generation behave similarly enough to the natural (spontaneous or induced) tumors to be useful for studies of hormonal influences on tumor growth, as the present work indicates.

b) Similar considerations apply to leukemia studies. With the widely used "chloroleukemia" of Shay, research is complicated by immunologic differences between grafts and hosts. The well inbred Fischer strain seems to be free of this complication, as does our W/Fu strain. Grafts take in 100 percent of Fischer rats and they do not regress. There is a remarkable difference between leukemias in Fischer and W/Fu rats; in the former, cures have been reported (*46*), whereas in the latter, chemotherapeutic agents merely prolong life, as they do in man, but do not completely control the disease. A wider assortment of spontaneous leukemias is available in the W/Fu than in the Fischer strain, some with extensive blood involvement, some with lymphosarcoma-type growth.

c) The available transplantable *mammotropic pituitary tumors* offer possibilities for research on both control of mammary cancer by specific inhibition of the mammotropes and physiologic studies of mammary-gland function.

d) As to *genetic and environmental factors* in the genesis of leukemia, the finding that the incidence of leukemia was not increased by extensive inbreeding deserves further analysis.

e) In *carcinogenesis* with hydrocarbons and radiations, the rat should be further explored because the spectrum of tumors which it yields is different from that of the mouse.

f) Similarly, in studies of *endocrine neoplasia*, the rat behaves differently from the mouse; *e.g.*, it fails to yield thyrotropic tumors following radiothyroidectomy and ovarian tumors after whole-body irradiation, which the mouse does. The implications of such species differences deserve to be explored.

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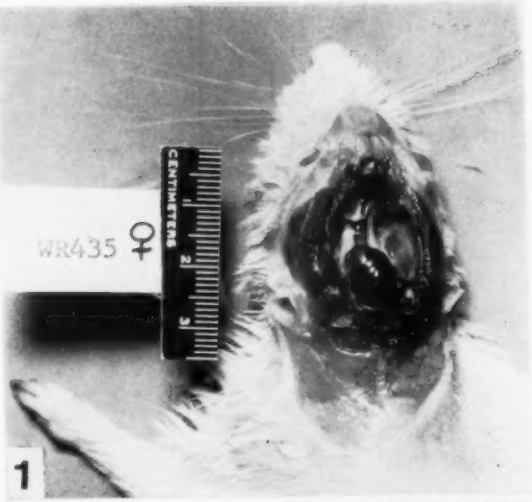
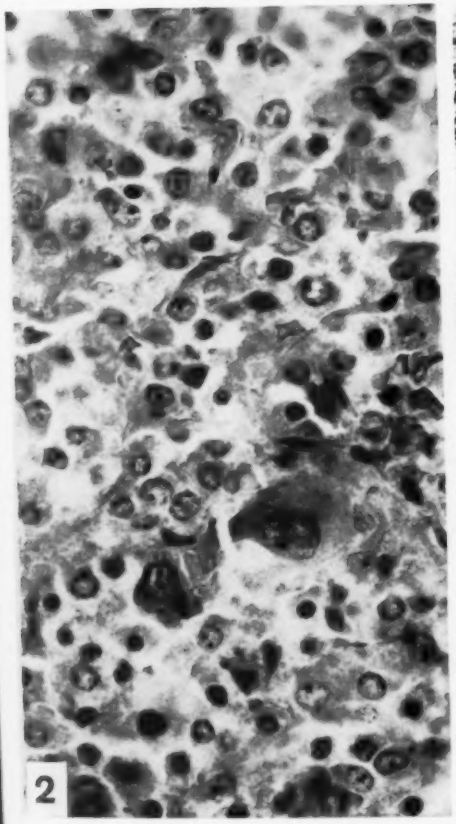
PLATE 89

FIGURE 1.—A spontaneous pituitary tumor in a 23-month-old female rat that had had 4 pregnancies during the first 18 months of life. The pituitary tumor measured 1.0 × 0.7 × 0.5 cm.

FIGURE 2.—The microscopic appearance of a spontaneous pituitary tumor from another aging female rat of 24 months of age that also had 4 pregnancies. Hematoxylin and eosin. × 600

FIGURE 3.—The mammary gland from the same rat as in figure 2, showing moderate hyperplasia with secretion. Hematoxylin and eosin. × 350





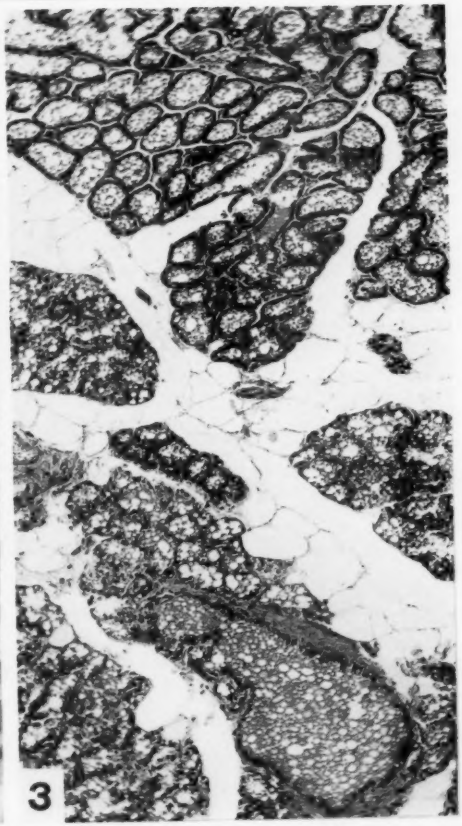


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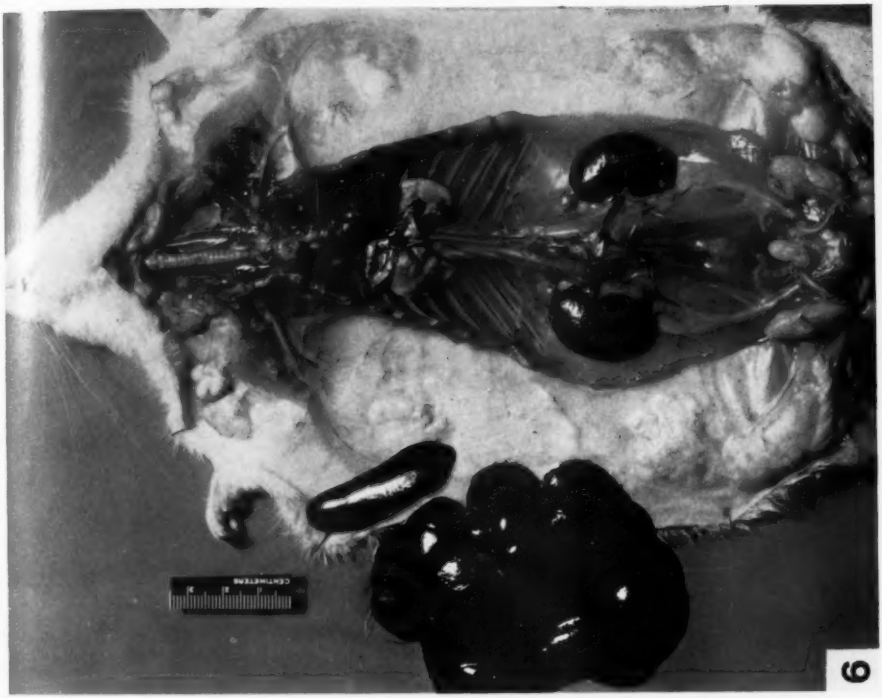
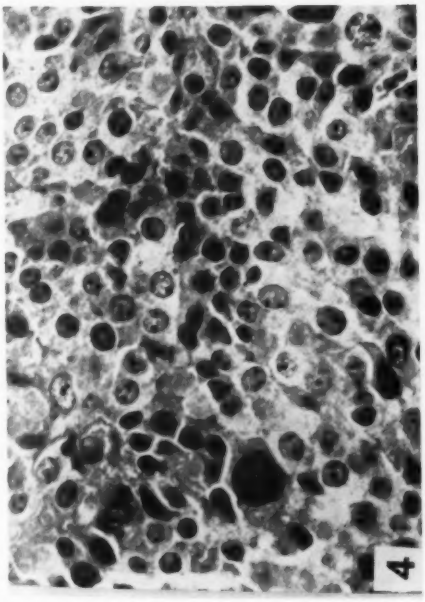
PLATE 90

FIGURE 4.—Microscopic appearance of second-generation graft of a spontaneous pituitary tumor (W-3; Ia) in an intact male (*see* table 2). Hematoxylin and eosin. × 600

FIGURE 5.—Mammary gland of the same animal as above. *Note* hypersecretory glands. The grafted tumor measured 2.0 × 1.5 × 1.0 cm. Hematoxylin and eosin. × 50

FIGURE 6.—Gross view of same animal. *Note* somatotropic effects in addition to the marked mammary-gland stimulation. This rat weighed 553 gm. (The average body weight of normal male rats of the same age is 280 gm.) The grafted pituitary tumor in the right thigh is not shown in this picture.





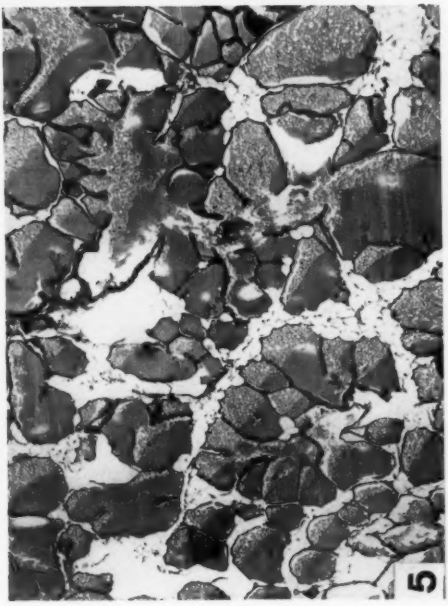


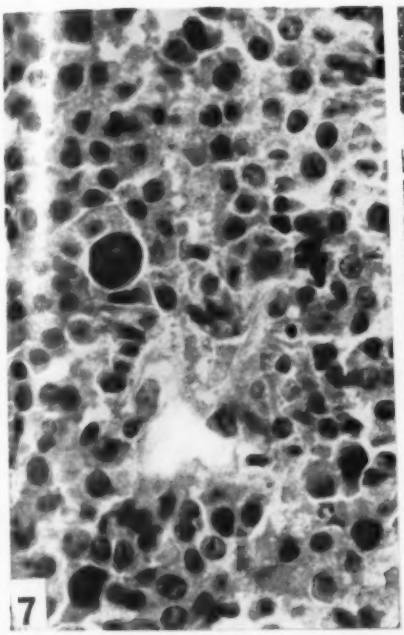
PLATE 91

FIGURE 7.—Sixteenth-generation graft of an estrogen-induced pituitary tumor in a castrated male Fischer rat. The tumor measured 5.0 × 3.0 × 1.5 cm. Hematoxylin and eosin. × 600

FIGURE 8.—Stimulated mammary gland of the same animal. Hematoxylin and eosin. × 50

FIGURE 9.—Adrenocortical tumor from 14-month-old female breeder. Hematoxylin and eosin. × 600

FIGURE 10.—Opposite adrenal of above, showing marked atrophic and degenerative cortex. The maximum diameter of this adrenal was below 2 mm. Hematoxylin and eosin. × 300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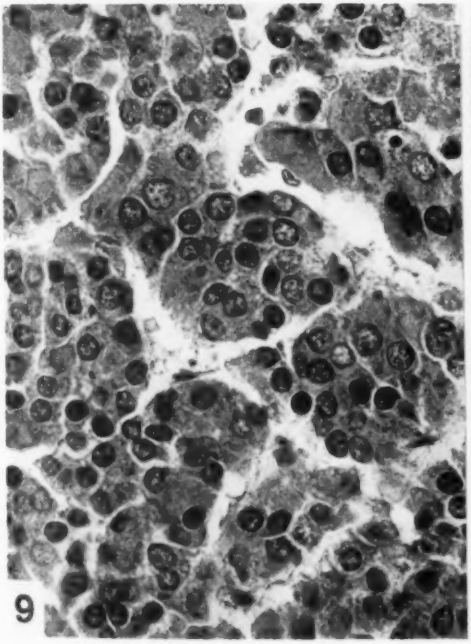
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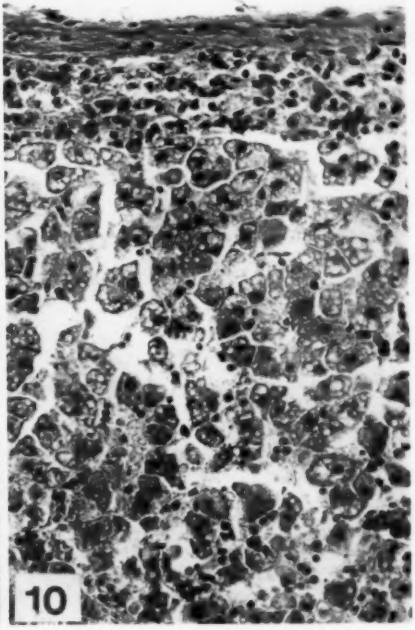
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PLATE 92

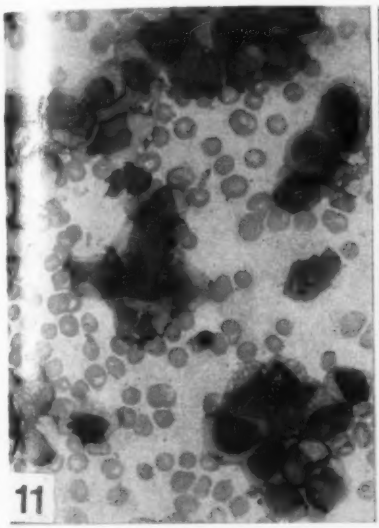
FIGURE 11.—Blood smear of a transplanted W/Fu leukemia showing many "blasts" and a nucleated red cell. Wright-Giemsa stain. × 350

FIGURE 12.—Greater magnification of above. × 1,100

FIGURE 13.—Blood smear of another strain of transplanted W/Fu leukemia. Wright-Giemsa stain. × 350

FIGURE 14.—Greater magnification of above showing azurophilic granules in cytoplasm. × 1,100

FIGURE 15.—Section of a lymph node from 30-month-old female breeder with malignant lymphoma. Hematoxylin and eosin. × 1,100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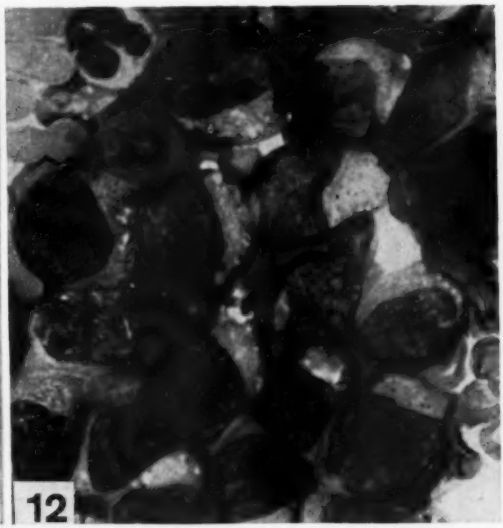
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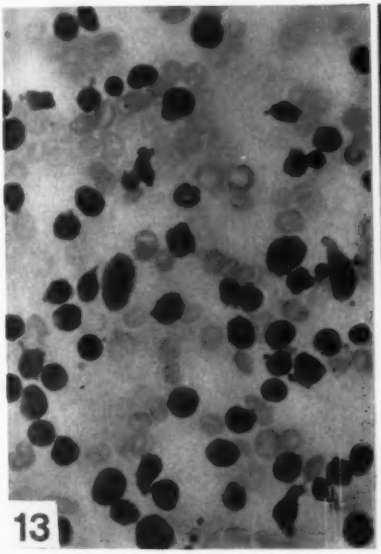
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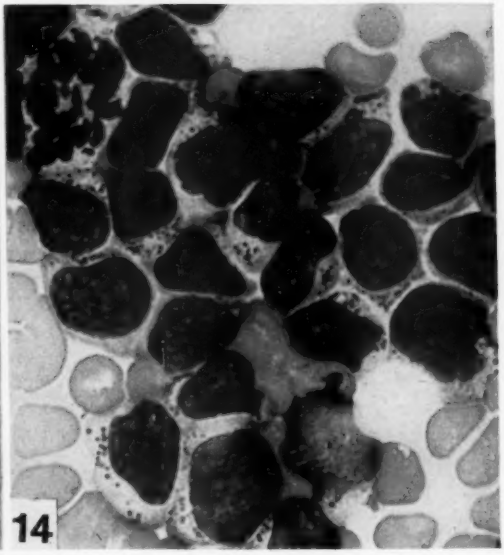
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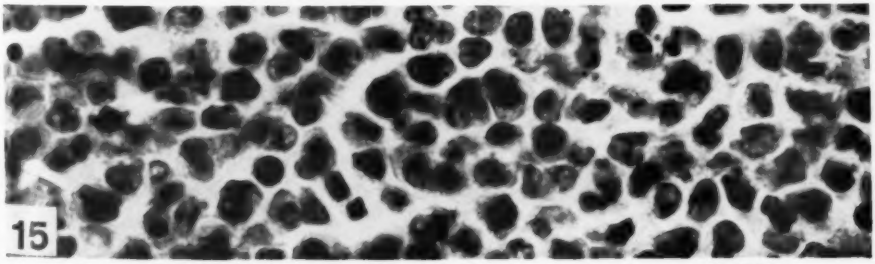
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Estrogen-Induced Kidney Tumors in the Golden Hamster. I. Biochemical Composition During Tumorigenesis [1, 2]

ROBERT F. McGREGOR, JOSEPH D. PUTCH, *and* DARRELL N. WARD,[3] *Biochemistry Department, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas*

SUMMARY

Male golden hamsters were treated with diethylstilbestrol to produce renal tumors. Analyses of total fat, protein, carbohydrate, and nucleic acid levels in both kidney and liver were made during the early stages of carcinogenesis (3 months), as tumors became detectable microscopically (8 months), and at a time when the tumors were well established (10 months). Appropriate controls were also examined. Appreciable changes in concentration of the foregoing constituents were not observed until the tumors were well established. Increased kidney weight appeared to be one of the earliest manifestations of tumorigenesis. Regression of the kidney tumors after 10 months of treatment with diethylstilbestrol followed by 1 month without estrogen was studied. Regression was complete in only 60 percent of the animals. For about one half of the animals, some factor other than removal of the excess estrogen appeared to be involved in the regression. Kidney function, as measured by urea clearance, was not affected after 8 months of treatment with diethylstilbestrol.—J. Nat. Cancer Inst. 24: 1057–1066, 1960.

THE INDUCTION of renal tumors in male golden hamsters by diethylstilbestrol (DES) was first described by Matthews, Kirkman, and Bacon (*1*) in 1947. Subsequent studies by Kirkman and Bacon (*2*, *3*) established the necessary conditions for development of the malignant transformation. Included in these studies were the effects of both natural and synthetic estrogens. Since then, rapid progress in complementary studies has enhanced our understanding of the endocrine interrelationships involved during the carcinogenic process. Thus, in one area, Kirkman and Horning have shown that progesterone and testosterone prevented the development of renal tumors in both intact males and gonadectomized animals of either sex (*4*, *5*) and have indicated the role of the adrenal-pituitary axis during tumorigenesis and regression (*6*). In another area, Horning has demonstrated by transplantation studies that the renal tumor, although it has all the histological characteristics of malignant

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[2] This investigation was supported by research grant C-4212 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] We are indebted to Dr. Jeffrey P. Chang of the Department of Pathology for the histological studies.

growths, even metastasizing via the lymphatics, never achieves full autonomy but remains hormonally dependent upon the level of estrogen (*7*). This dependence has been shown to exist after 4 years of serial grafting.

Horning, using unilateral nephrectomy (*8*), and Ising, using unilateral ureterectomy (*9*), studied the effect of the functional state of the kidney upon the course of tumor development. In both studies, the disruption of normal function led to earlier tumor formation and greater growth in the affected kidney.

A study of the biochemical patterns associated with tumorigenesis in the hamster kidney has not been reported. Therefore the following study has been made to supplement the observations of others and to provide information for additional studies we plan to make on this hormone-dependent carcinogenic process. The present report also includes some preliminary observations on tumor regression in the absence of DES.

The biochemical parameters selected for study were lipide, protein, carbohydrate, and nucleic acid concentrations. These constituents were measured for both kidney and liver during the precancerous phase (3-month group), the early period of tumor appearance (8-month group) and, finally, the time when there were well-established tumors (10–12-month group). Body, testis, liver, and kidney weights were also measured for each animal killed.

MATERIALS AND METHODS

Male golden hamsters, 6 to 8 weeks old, were obtained from the Golden Nugget Hamstery, Maynard, Massachusetts. The animals were housed in air-conditioned animal quarters, 5 to 8 animals per cage, and fed Fox chow and water *ad libitum*. Within 2 weeks after the animals arrived, a pellet containing 20.0 to 27.0 mg. of pure diethylstilbestrol (DES) was implanted subcutaneously on the back under ether anesthesia. At 8 to 12 weeks from this time, another pellet of DES was implanted to insure continuing levels of stimulation (*7*). A group of animals was killed 3 months after the first implantation, another group at 8 months, and the last group at a time ranging from 10 to 12 months after the first implantation. The testicular weight of some of the animals receiving 2 pellets had increased after 10 to 12 months on the experiment. Examination at autopsy showed that the pellets were completely absorbed. Consequently, a group at the 8-month level was implanted with a third pellet and carried for 3 to 4 more months. These animals were designated as "3-pellet, 10 to 12-month animals" and were killed at various intervals 10 to 12 months after treatment with DES was started in order to check tumor incidence. In this and a number of subsequent experiments in which 3 pellets were implanted a 100 percent incidence was obtained after 10 months of treatment, which afforded a reliable comparison with the "regression" groups. Chemical changes showed no variations during this period, however, so the values have been pooled for simplicity of presentation.

At the selected time, the animals were weighed and placed under ether anesthesia. The abdomen was opened, the vena cava was severed between the liver and diaphragm, and after bleeding had ceased, the testes, liver, and kidneys were removed and weighed. All abnormal-looking areas were excised and saved for histologic examination. From liver and kidney tissues 10 percent homogenates in cold 0.007 M arsenate buffer were prepared, as described by Emanuel and Chaikoff (*10*). Homogenization was effected with a Potter-Elvehjem homogenizer in an ice bath. To each of four 2.0 ml. aliquots of the 10 percent homogenate were added 6.0 ml. of cold 10 percent trichloroacetic acid (TCA). The samples were allowed to stand overnight in the refrigerator before centrifugation. Two of the samples were reserved for total-carbohydrate analysis, after a modification of the method of Trevelyan and Harrison (*11*). The supernatant was used to measure the acid-soluble carbohydrates (designated as "TCA(CHO)" in tables 1 and 2), and the residue was used for analysis of "residual carbohydrates," *i.e.*, those liberated by extraction with 60 percent H_2SO_4 overnight and hydrolyzed with 5N H_2SO_4 at 100° C. for 1 hour [designated as "H_2SO_4(CHO)" in tables 1 and 2]. Both supernatant and residual carbohydrate were measured by the anthrone procedure of Handelsman and Sass (*12, 13*) and are reported as mg. or μg. of glucose according to the range of concentration.

The TCA precipitates of the remaining duplicate samples were extracted once with 95 percent ethanol and twice more with Bloor's reagent (3:1 alcohol-ether) at 70° C. for 10 minutes. The total lipide was determined gravimetrically. The residue after the lipide extraction is essentially the same as that obtained in the Schneider procedure for estimation of nucleic acids (*14*). At this point, 5.0 ml. of 5 percent TCA was added to the residue and the nucleic acids were extracted at 90° C. for 15 minutes. The centrifuged residue was resuspended and washed with 5 percent TCA, and, finally, the wash was combined with the original extract and made to a volume of 10.0 ml. Deoxyribonucleic acid (DNA) was determined by the Burton-Dische method (*15*) and ribonucleic acid (RNA), by the orcinol method of Mejbaum (*16*).

Separate duplicate aliquots from the original homogenate were taken for determination of nitrogen by the micro-Kjeldahl method with the procedure of Miller and Houghton (*17*), and from this the protein concentration was estimated; the factor of 6.25 was used for conversion of nitrogen to protein.

A group of 8-month DES-treated hamsters was used for urea clearance studies. The animals were placed in individual metabolism cages for 24 hours without food or water, and the urine was collected under toluene in a graduated cylinder. Determinations were made of urea nitrogen of both blood and urine, by the Conway microdiffusion method (*18, 19*), specific gravity, by a modification of the method of Barbour and Hamilton (*20*), and *p*H, which was read directly on a *p*H meter.

The literature at present does not discuss in detail the preparation of DES pellets for implantation. Although some authors suggest melting

the DES in capillary tubes, we found it difficult with this method to exclude enough air to prevent oxidative decomposition, and the pellets obtained were generally smaller and of less uniform size than desired. Because of the difficulty of obtaining satisfactory pellets, a description of our process may be useful. Pure DES pellets prepared by a hand press are often too brittle and difficult to handle with a forceps at implantation and sometimes crumble under the skin. Some of our pellets were prepared by hand-pressing the DES after it had been precipitated from a saturated solution in alcohol by the addition of water, which produces a larger crystal aggregate that has better binding properties. The pellets were dried to constant weight before implantation. Our current method of preparing uniform pellets is as follows: To 3.0 gm. of DES powder in a mortar, 0.5 ml. of ethanol and 2.0 to 2.5 ml. of water are added, and the mixture is ground to a smooth paste, which is then extruded through a 3 mm. orifice (a pellet-press die) to produce long cylinders of DES. The DES cylinder is cut into the proper pellet lengths, 20 to 30 mg., by a razor blade; the pellets are air-dried for several days and finally dried to constant weight in an oven at 110° C. The pellet prepared in this manner is stronger when handled by forceps than any other type we have prepared.

RESULTS AND DISCUSSION

The young animal tolerates the DES pellet well—our losses are negligible until the 8-month period. From 8 months on, the treated animals occasionally lose weight and display some alopecia and emaciation. At this time they are susceptible to attack by their cage mates, and deaths more often are due to this than to the renal tumor. The control animals gain weight steadily until they weigh 150 to 160 gm. at approximately 20 weeks of age. In a few instances, some of our normal animals (12 months and older) have achieved weights of 220 gm. The DES-treated animals do not gain weight as well as the controls (table 1); they average some 20 to 30 gm. less than the controls of the same age. Other authors, in comparison, report much smaller weight gains (*21*, *22*). Moreover, we have never observed the winter regression of testicular weight reported by Koneff, Simpson, and Evans (*22*). The 6-month controls, reported in the study of the California group, show that this winter regression is of considerable magnitude, with testicular weights as low as 485 mg. In spite of high levels of DES (90 mg. in some males) no renal tumors were observed in their treated animals. Since it is inconceivable that the tumors were overlooked, the failure of tumor development coupled with unusual testicular response may be explained by nutritional or environmental differences. It has long been known that food intake is very important in determining the response to estrogens (*23*). In addition, their study of the pituitary gland revealed different histological changes than those reported by Vazquez-Lopez (*24*) who, however, had noticed

a large secondary "deposit" in the kidney. In our studies the incidence of kidney tumors at 8 months is high, and, if estrogen levels are maintained, the incidence after 10 months is 100 percent (table 1). (For the incidence when the level is not maintained *see* the 10 to 12-month 2-pellet group.) The tumors in each group were confirmed histologically.

The results of the biochemical-composition measurements are summarized in table 1. Similar values from animals after a 1-month regression period following removal of DES are listed in table 2. Table 1 shows that the control animals have higher protein concentrations in the liver and kidneys than the corresponding DES-treated animals. Kidney weights are greater among the DES-treated animals as originally shown by Kirkman and Bacon (*3*). This increased kidney weight is already detected during the 3d month of treatment (3-month groups, table 1), although the differences are not great—calculated as percentage of body weight the differences are more apparent.

The livers of control animals appear to have greater amounts of acid-soluble carbohydrate than those of 10 to 12-month DES-treated animals. Since this carbohydrate is principally liver glycogen, the data suggest that the ability of the liver to store glycogen may be somewhat impaired after prolonged estrogen treatment. The carbohydrate content of the kidney was essentially the same in both control and treated groups.

Lipide measurements for both kidney and liver generally showed a lower value in the DES-treated animals, but again no pronounced and uniform differences were observed.

The most striking difference in ribonucleic acid concentration was noted in the animals in the oldest group receiving continued high levels of estrogen (table 1, 3-pellet group). Both kidney and liver RNA values were suppressed, whereas in the oldest control animals, by contrast, there was an appreciable increase in RNA concentration. The DNA values were slightly higher in the livers of DES-treated animals (table 1). However, in the kidney the DNA values were similar in both control and treated animals, which is quite different from what is generally observed in affected tissue during chemically induced carcinogenesis (*25*, *26*), namely, an increase in DNA concentration during tumor production. This increase, coupled with a decrease of cytoplasm and RNA as the cell population progresses toward that characteristic of the tumor, leads to decreased RNA/DNA ratios (*26*). Although the RNA/DNA ratio in the kidney of controls increased with time, the ratio in the treated animals remained essentially constant (table 1). The ratio in the liver was constant for all control groups except the 10 to 12-month animals (table 1), in which the very high RNA concentration raised the ratio considerably. The liver RNA/DNA ratio in the DES-treated animals was constant at approximately 1.75 in all groups, which is less than that obtained in the liver of the controls.

From an inspection of the concentration values in table 1, the most striking observation is the absence of any pronounced changes in terms of the parameters measured. Those changes that were detected by

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Table 1.—Measurements obtained during renal-tumor induction with diethylstilbestrol (DES)*

	Control groups (no treatment)				DES-treated groups			
	0-Month	3-Month	8-Month	10-12-Month	3-Month	8-Month	10-12-Month 2-pellet	10-12-Month 3-pellet
Number of animals (n)	10	5	13	9	17	12	18	15
Tumor incidence	0	0	0	0	0	9/12	10/18	15/15
Final body wt. (gm.)	105 ± 4.1	151 ± 16.5	153 ± 3.9	159 ± 8.3	120 ± 4.9	131 ± 6.4	122 ± 4.0	133 ± 5.4
Testicular wt. (mg.)	3,010 ± 63.3	2,535 ± 59.8	2,420 ± 171.7	2,930 ± 119.3	159 ± 6.3	245 ± 32.1	1,500 ± 191.0	237 ± 18.3
Liver wt. (gm.)	4.84 ± 0.4	7.45 ± 1.08	7.31 ± 0.31	8.60 ± 0.65	5.82 ± 0.35	7.34 ± 0.34	6.13 ± 0.37	7.01 ± 0.41
Liver protein (%)	19.75 ± 0.4	18.34 ± 0.32	19.64 ± 0.42	18.74 ± 0.35	17.94 ± 0.16	17.88 ± 0.48	17.78 ± 0.61	17.38 ± 0.39
Liver lipide (%)	8.1 ± 0.5	7.3 ± 0.71	9.6 ± 0.61	9.4 ± 0.67	6.3 ± 0.17	7.8 ± 0.35	9.7 ± 0.24	7.4 ± 0.59
Liver DNA (mg./100 gm.)	265 ± 8.5	222 ± 15.6	234 ± 5.8	217 ± 13.3	311 ± 17.2	282 ± 19.9	328 ± 18.2	274 ± 13.2
Liver RNA (mg./100 gm.)	532 ± 14.2	535 ± 21.0	491 ± 24.7	704 ± 55	540 ± 32.3	597 ± 27.2	578 ± 21.2	480 ± 29.5
RNA/DNA ratio	2.01	2.41	2.10	3.24	1.73	2.12	1.76	1.75
TCA(CHO) (mg./100 mg.)	5.12 ± 0.2	3.79 ± 0.43	5.50 ± 0.31	4.18 ± 0.41	4.25 ± 0.26	3.49 ± 0.34	3.85 ± 0.42	2.66 ± 0.31
H_2SO_4(CHO) (μg./100 mg.)	517 ± 24.7	355 ± 17.9	502 ± 33.2	461 ± 26.3	353 ± 14.8	330 ± 30.6	409 ± 46.7	242 ± 23.3
Kidney wt. (gm.)	0.808 ± 0.019	0.959 ± 0.089	1.120 ± 0.069	1,180 ± 0.12	1.13 ± 0.05	1.36† ± 0.061	1.23 ± 0.083	2.20† ± 0.339
Kidney protein (%)	17.37 ± 0.48	16.23 ± 0.79	15.51 ± 0.59	17.74 ± 0.59	15.31 ± 0.40	13.65 ± 0.80	16.66 ± 0.50	13.65 ± 0.55
Kidney lipide (%)	7.6 ± 0.25	6.2 ± 0.40	9.2 ± 0.64	9.8 ± 0.47	5.8 ± 0.22	7.8 ± 0.52	10.2 ± 0.85	7.6 ± 0.75
Kidney DNA (mg./100 gm.)	546 ± 12.7	444 ± 39.3	373 ± 27.1	426 ± 26.3	427 ± 19.9	390 ± 27.5	521 ± 26.9	358 ± 16.3
Kidney RNA (mg./100 gm.)	391 ± 12.0	396 ± 15.6	327 ± 10.2	516 ± 35	343 ± 19.2	350 ± 11.8	372 ± 9.4	251 ± 10.1
RNA/DNA ratio.	0.72	0.89	0.88	1.21	0.80	0.90	0.71	0.70
TCA(CHO) (μg./100 mg.)	152 ± 9.8	100 ± 5.4	123 ± 8.0		131 ± 7.8	187 ± 27.2	276 ± 43.9	135 ± 16.0
H_2SO_4(CHO) (μg./100 mg.)	209 ± 24.7		154 ± 11.9	260 ± 54.3	137 ± 2.7	170 ± 11.8	233 ± 8.5	157 ± 11.6

*Values represent the mean of *n* animals ± standard error of the mean.

†Excluding one pair of tumorous kidneys weighing over 18.0 gm.

chemical measurements were found only after the tumors were well established. In two instances (*see* table 1) tumorous kidneys weighing over 18 gm. were found, but even in these unusual instances the DNA concentration did not attain that of a highly cellular tumor due to the presence of numerous small cysts contributing to the total organ weight. In fact, DNA concentration values were slightly below the DNA averages listed in the table.

If the values in table 1 are viewed in terms of percentage increases over initial quantities in the entire organ, certain differences are more apparent than from a simple examination of the table. For instance, the total percentage increase in kidney weight was from twofold to fourfold greater in the DES-treated animals than in the controls. The DNA increase in the kidney very nearly paralleled this increase in weight for DES-treated animals, while total DNA remained essentially constant for the controls.

The growth pattern of the liver may be ascertained by calculation from the figures in table 1. Increases of liver weight were always higher in the controls than in DES-treated animals. However, the liver DNA showed an opposite relationship, namely, the percentage increase in DNA of the entire liver was always greater in the DES-treated animals than in the controls. The RNA of the entire organ pointed to a more variable growth pattern. With prolonged DES treatment, decreased total RNA, as compared to the increase of total RNA in the older controls, was very marked.

Tumor-Regression Studies

For the preliminary studies on tumor regression 20 animals that had been treated with DES for 10 months (3-pellet group) were examined by laparotomy for kidney tumors, after which the DES pellets were removed surgically. Every 3 or 4 days 2 animals were killed and examined for signs of tumor regression. Complete regression was observed only in 1 of 2 animals carried through an entire 1-month "regression" period following DES removal. Then, a second experiment was carried out with 10 animals treated for 10 months with DES (3-pellet group). Those DES pellets that remained were removed surgically, and after 1 month all animals were killed, the animals were examined grossly and histologically for tumors, and the kidney and liver were analyzed chemically, as described. Autopsy confirmed the removal of all DES pellets, also indicated by the recovery of testicular weight (table 2). The livers of all animals showed lobe rounding, and 80 percent had grossly visible cysts. In contrast, the livers of DES-treated controls (3-pellet group, 10–11 months) with a 100 percent tumor incidence appeared normal. This indicates that DES withdrawal in some manner favored liver-cyst formation. In other experiments involving 10 to 12-month DES-treated animals, an isolated liver cyst has been seen occasionally, but this in no way compares with the severity of damage observed in "regression" animals.

The values obtained from the analyses of liver and kidney of the "regression" animals have been tabulated in table 2 according to whether tumors were present or absent. Comparison of these figures with those in table 1 indicates that the values are essentially the same as those obtained with the 10 to 12-month 3-pellet group. The only exceptions are a lower liver RNA value in the animals still bearing kidney tumors and lower kidney weight in those animals with complete regression. However, kidney weight in the latter animals was still about 15 percent greater than in untreated controls of the same age (table 1).

TABLE 2.—Measurements obtained during regression study after 10 months of diethylstilbestrol treatment and 1 month without*

	Tumors still present	Tumors absent (regressed)
Number of animals (*n*)	4	6
Final body wt. (gm.)	133 ± 4	114 ± 6.5
Testicular wt. (mg.)	1,620 ± 0	888 ± 129.8
Liver wt. (gm.)	7.38 ± 0.35	5.72 ± 0.60
Liver protein (%)	14.57 ± 1.01	18.12 ± 0.39
Liver lipide (%)	8.6 ± 0.65	7.8 ± 0.73
Liver DNA (mg./100 gm.)	240 ± 14.0	296 ± 10.2
Liver RNA (mg./100 gm.)	348 ± 15.0	517 ± 44.5
RNA/DNA ratio	1.45	1.75
TCA(CHO) (mg./100 mg.)	1.88 ± 0.22	2.93 ± 0.57
H_2SO_4(CHO) (μg./100 mg.)	239 ± 28.0	282 ± 10.2
Kidney wt. (gm.)	1.95 ± 0.20	1.36 ± 0.008
Kidney protein (%)	12.18 (2)	16.95 ± 0.39
Kidney lipide (%)	11.6 ± 0.50	8.1 ± 0.90
Kidney DNA (mg./100 gm.)	383 ± 16	422 ± 24.5
Kidney RNA (mg./100 gm.)	279 ± 29	323 ± 16.3
RNA/DNA ratio	0.73	0.77
TCA(CHO) (μg./100 mg.)	171 ± 6	156 ± 3.7
H_2SO_4(CHO) (μg./100 mg.)	173 ± 6.5	255 ± 35.1

*Values represent the mean of *n* animals ± standard error of the mean.

In the experiment presented in table 2, 60 percent of the animals showed complete tumor regression. In other experiments the "regression" period (without DES) were continued for 2 months and approximately the same percentages were observed, namely, 50 percent showed complete regression and 50 percent still had tumors. Thus our studies indicate some factor(s) other than removal of the excess estrogen is involved in regression. Further work concerning this point is planned.

Kidney Function After DES Treatment

Since Ising (*9*) and Horning (*8*) have shown that the functional state of the kidney influences the rate of tumor production, an experiment was carried out to determine whether prolonged treatment (8 months) by DES affected kidney function at the point when tumors begin to be microscopically detectable. Kidney function was measured in terms of urea clearance. The results are shown in table 3. Neither urea clearance

TABLE 3.—Kidney function test after 8 months of treatment with diethylstilbestrol

Animals	Urine (*mean 24-hour values*)			Urea urine/ blood ratio	Urea clearance (*ml./hr.*)
	Volume (*ml.*)	*p*H	Specific gravity		
Normal (4 hamsters)	5. 7	6. 9	1. 037	18. 7	3. 4
DES-treated (4 hamsters)	5. 1	6. 5	1. 040	15. 0	3. 2

nor urine volume, *p*H, or specific gravity appeared appreciably different in the DES-treated animals than in the controls. Thus, by these criteria, kidney function is not altered after 8 months of treatment with DES.

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Mammalian Chromosomes *In Vitro*. XII. Experimental Evolution of Cell Populations [1, 2]

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SUMMARY

Strain L–P59, a subline of strain L clone 929 of the mouse, had a chromosome modality of 64 to 66 with 13 or 14 metacentrics. After the population had been treated with colchicine for 48 hours, cells in the modality zone (stem cells) were greatly reduced in number. The population was predominantly occupied by high polyploids and, in the stemline zone, by genomes less frequently observed in the original population. These were considered as metabolically second-rate genomes. The stem cells, as expected, regained dominance after several subcultures. After prolonged colchicine treatment, the cultures either shifted completely to double-stemline numbers or changed to genomes that were rarely found in the parental population. The modality dropped to below 60, and many "monstrous" chromosomes were recorded. The double-stemline strain was apparently the result of polyploidization of the stem cells; but the rare genomes were interpreted as those that escaped colchicine poison. While all the vigorous genomes were repeatedly poisoned by colchicine whenever they entered mitosis thereby becoming giant cells which lost the ability to give rise to cell lineage, these rare genomes, due to their inferior metabolic activities, did not divide during the period of treatment, thus escaping injury. Growth assays confirmed that these cells grew much slower than the parental types. Long-term colchicine treatment on cells *in vitro* may be used as a method for selecting metabolically inferior mutant genomes whose isolation would be otherwise difficult.—J. Nat. Cancer Inst. 24: 1067–1093, 1960.

IT IS a well-established fact that in any cell population *in vitro*, genetic polymorphism is the rule rather than the exception. Prominent among the variations is the presence of polyploid cells. In primary cultures or in early transfers of normal tissues where diploid cells predominate there is always a small fraction of tetraploid and octoploid elements. Levan and Biesele (*1*) observed a gradual increase of polyploids in the cultures of certain mouse embryonic tissues. They postulated, as did Hsu and Klatt (*2*), who observed a similar phenomenon in rat tumor cultures,

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that polyploids probably have some adaptational advantages over the diploids for the environment *in vitro*.

In established cell strains, where true diploid and tetraploid cells rarely exist, the stemline cells (or stem cells) are usually heteroploid with chromosome numbers in between the diploid and the tetraploid limits. Again we often observe a small fraction of higher polyploids that are in reality secondary (double-stemline) and tertiary (multiple-stemline) heteroploids. However, from generation to generation the proportion of the double-stemline cells does not significantly change. A question thus arises: Are the cells with double-stemline chromosome number metabolically inferior? If so, in what respect? Is it because they multiply at a slower rate due to possible imbalance in their genetic material or simply because of physical reasons? It seems that under a given environmental condition an equilibrium between the stem cells (s) and the double-stemline cells (2s) is always maintained. Can this equilibrium be broken or altered? Undoubtedly 2s cells are formed through mitotic irregularities of the s cells. We know that 2s cells are capable of entering mitosis because we observe them at metaphase. Unless each mitosis of a 2s cell would lead to the death of the filial elements, it should be possible to obtain cell lines consisting purely of secondary heteroploids from mixoploid populations.

Makino (*3*) enumerated evidences to support his thesis that the stem cells are the strongest, the most vigorous, and the most resistant of all the genomes in the cell populations. After various kinds of stresses, the cells that recovered were invariably the stem cells, not the polyploids or other aberrant types. However, Kaziwara (*4*) showed that, with appropriate pressure, populations may change their structure to a double-stemline dominated condition, but none were completely converted. Furthermore, after the removal of the pressure, some of the populations returned to their original composition (*4*, *5*). The Makino group (*6*) later also modified the stemline concept to one that is more flexible.

A number of investigators attempted to polyploidize ascites tumors of the mouse by colchicine treatment. Levan (*7*), for instance, changed the populations of the Landschütz mouse tumor to near 90 percent polyploids. But the populations did not stabilize at that level, especially after retransplantation. Perhaps it is difficult to perform single-cell inoculation with polyploid cells to secure stable polyploid lines, but it should not be a serious problem to clone polyploid cells *in vitro*. In fact, Biedler (*8*) succeeded in obtaining an octoploid clone from a population of Sarcoma 180 by the Puck technique. The chances of isolating polyploid clones should greatly increase if the populations could be pretreated with a proper concentration of colchicine.

Cell populations do not only contain stemline and double-stemline elements. Within the s range there may be numerous genomes, some conceivably less vigorous than others. Some of these "mutants" may be so inferior that they do not have a fighting chance to compete with the stem cells under ordinary conditions. In order to recover the most inferior cells, the stem cells and the slightly inferior genomes must be completely

eliminated. The use of antimetabolites may also interfere with the metabolism of the feeble genomes. Colchicine treatment, on the other hand, may be more desirable because colchicine is a spindle poison which affects only cells in mitosis. If metabolically inferior genomes do not enter mitosis during the period of colchicine treatment, they may escape injury whereas the stem cells would be polyploidized repeatedly. Usually high polyploids are capable of entering mitosis, but they either fail to divide or fuse after cleavage even in the absence of colchicine, thus becoming giant cells in either case (unpublished data). The giant cells, like those formed after irradiation, would survive for a considerable period before degenerating. Thus when a cell population *in vitro* is subjected to the influence of a nonlethal dose of colchicine, several possibilities may develop:

1. Most of the stem cells may be affected at least once and the population will shift to a high proportion of 2s cells. When colchicine is removed, the population will gradually return to the original composition by the growth of the unaffected s cells.
2. All the stem cells may be affected at least once; the populations thus may consist only of 2s cells.
3. All the stem cells may be repeatedly poisoned, and only the inferior genomes that did not enter mitosis during the period of treatment will escape poisoning. The resulting populations thus may be strains of metabolically inferior mutants.
4. After overtreatment with colchicine, no cell type will survive unless colchicine resistance develops.

The present communication presents examples to demonstrate that populations *in vitro* can be altered experimentally. It also suggests a method by which slow-growing mutant genomes can be isolated from genetically mixed cell populations.

MATERIALS AND METHODS

In our laboratory strain L-P55 of the mouse has been maintained in 2 entirely different stocks since 1957, by different workers and in medium from different containers. The second stock was carried as a reserve in only 2 culture vessels at a time, and no experiments were made with these cells. The chromosome features of L-P55 described in our previous papers (*9*, *10*) were based on the cells of the main stock. A laboratory disaster that occurred late in 1958 wiped out the main stock and we were obliged to propagate the reserve cultures as a replacement. It was immediately found that both metabolically and cytologically the reserve stock was different from the original (*11*). Since the original L-P55 is no longer in existence, we hereby redesignate the strain presently in use as L-P59, the cytological characteristics of which will be described in this report.

The stock cultures of L-P59 have been carried in McCoy's 5a medium (*12*) with 10 percent horse or calf serum since February, 1959. Prior to that time they were carried in Eagle's medium. Subcultures were generally made once a week with the aid of a fishtail rubber policeman

without the use of trypsin. No change of medium was found necessary between subcultures.

Development of sublines.—The L-P59 cells were seeded in Owen's ovals with regular 5a medium for 1 or 2 days, then medium containing colchicine was applied. Colchicine solution was added to the medium to a final concentration of 2 μg. per ml. The cells were treated with colchicine medium for periods ranging from 2 days to 21 days. Those treated for 21 days received an intermittent removal of colchicine for 1 week.

After the final removal of colchicine, the cultures were permitted to grow in regular 5a medium and the survivors, after reaching a certain population size, were propagated in Blake bottles.

Growth curves.—The method used for conducting growth experiments was essentially the same as previously described (*11*) with the following minor modifications:

1. The growth medium was McCoy's 5a medium with 10 percent calf serum instead of Eagle's medium with 5 percent horse serum.
2. A 1 percent stock trypsin solution was prepared by dissolving "Trypsin 1 : 300" (Nutritional Biochemicals, Cleveland, Ohio) in Gey's balanced salt solution. It was then filtered and stored in small quantities in a deepfreeze. Before use, a bottle was thawed and the solution was mixed with 5a growth medium to reduce the final trypsin concentration to 0.2 percent. This solution was found satisfactory for practically all types of cells carried in our laboratory, with little cellular damage for as long as 1 hour after treatment. In the experiments reported here, the trypsin solution was used in seeding as well as in final cell counts.

Chromosome techniques.—The cytological techniques used in this study were the same as described previously (*9*).

DNA determinations.—A modification of Ceriotti's indole technique was used for determination of deoxyribonucleic acid (DNA) (*13*). Essentially the technique consisted of a hot perchloric acid extraction of the cells followed by a hot hydrochloric acid-indole treatment of the sample. Chloroform extraction of the resulting deoxyribose-indole complex removed interfering color complexes. In our laboratory the chloroform extraction was accomplished in conical centrifuge tubes, which were stirred by applying the tube tips to a stopper stirrer. The stirrer was constructed with a #8 rubber stopper having a slightly eccentric ¼-inch hole through its long axis. One end of this stopper hole was pushed onto the shaft of a ⅓₀-hp. motor and a ⅛ × ¼-inch piece of cork was pressed ⅛ inch into the other end of the stopper. The cork prevented tube seizure by the rubber. The contents of conical centrifuge tubes were swirled vigorously when held lightly in the fingers and applied lightly to the cork-bottomed hole in the stopper. Finger distribution along the tube controlled the swirling. The resulting milky emulsion was held for 5 seconds. This emulsion separated readily and the chloroform was easily removed with a water aspirator and Pasteur pipette.

Standard curves obtained with salmon sperm DNA (California Foundation for Biochemical Research, Los Angeles, Calif.) gave consistently linear values, and practically superimposable curves were obtained when 5 to 20 μg. per ml. was used for over a year. Standard and experimental

values from this indole technique were closely comparable to those obtained with the diphenylamine technique of Burton for these mammalian cells. Values reported herein for each cell line were averages of at least 10 determinations, which showed a variability of not more than 1 to 3 percent. This indole technique was felt to be superior to the diphenylamine because (1) it was faster, (2) very low blank values were regularly obtainable, (3) smaller cell samples could be utilized, and (4) sensitivity was tenfold greater. It should be noted that this procedure was devised for mammalian-cell material about 1 mg. in magnitude. We found that on a cell-number basis the best results were obtained with 1×10^6 cells. It was our experience that the most important step in determining the DNA levels of a culture of mammalian cells was the cell-counting step. The accuracy of cell counting may suffer from either nonuniform sampling or the presence of intact dead cells. A magnet-stirred suspension and careful speed would minimize the former, and the use of phase optics and experience would effectively reduce the latter.

RESULTS

General Description of Colchicine Effects

After 24 hours of colchicine treatment, the L cells usually rounded up considerably; many exhibited rather thick cytoplasmic processes with broad tips. As the colchicine effect continued, arrested mitoses greatly increased and a number of them eventually returned to interphase without anaphasic movement. These cells were characterized by large, roundish and flat cell bodies with lobated nuclei or with numerous micronuclei (*cf.* figs. 3 and 4). After a few days of colchicine treatment, the populations consisted of numerous large, flattened cells that resembled epithelial elements. Removal of colchicine enabled the unaffected cells to divide regularly, but the cells that had been arrested one or more times generally showed abnormal mitoses of various types. At this time extremely tiny cells were not uncommon. Presumably these were the results of multipolar divisions and other mitotic anomalies. The giant cells, after several failures of cytokinesis, degenerated and gradually decreased in number. Results of subsequent treatment with colchicine repeated what happened during the first treatment. After the last treatment with colchicine, very few cells of the original size could be noticed by scanning through the culture vessels. Two or 3 weeks later, scattered small cells were sometimes noted and were propagated to produce sublines.

The Parental Strain L-P59

Cells of strain L-P59 resembled those of other L lines in general cultural characteristics and in cellular morphology. Compared with the typical fibroblasts they were much shorter and broader. Large, polyploid cells invariably occurred in every culture with a frequency around 1 percent.

TABLE 1.—Mean values for chromosomal characteristics of strain L-P and its derivatives Amy, Barbara, and Carolyn

Cell line	Sampling time	Total chromosomes	Metacentrics	Subtelocentrics	D chromosome	Number of cells
L-P55*	Spring 1958	67. 9	12. 9	3. 8	1. 2	50
	Fall 1958	67. 3	13. 3	3. 2	0. 9	50
L-P59	Jan. 1959	64. 1	13. 9	2. 0	0. 6	50
	June 1959	63. 7	13. 1	2. 5	1. 0	50
Amy	June 1959	128. 5	24. 3	5. 4	1. 9	25
Barbara	June 1959	60. 0	14. 2	2. 9	0. 0	25
	July 1959	57. 9	15. 6	2. 4	0. 0	25
	Aug. 1959	55. 1	17. 9	2. 8	0. 0	25
Barbara-1	July 1959	56. 4	16. 8	2. 7	0. 0	25
Barbara-2	Aug. 1959	56. 8	18. 3	2. 6	0. 0	25
Carolyn	July 1959†	64. 3	12. 5	2. 5	0. 6	100
	Aug. 1959†	64. 4	13. 7	2. 5	0. 5	50
	Aug. 1959	63. 3	12. 9	2. 4	1. 0	25

*Data from Hsu and Kellogg (*11*).

†Calculations were based on equal number of cells with and without the D chromosome. The data are therefore somewhat biased, especially for the frequency of D chromosome.

The main cytological difference between strains L-P55 and L-P59 was the total chromosome number. L-P55 had a mean chromosome number of 67 to 68, whereas L-P59 had approximately 64 (table 1).

The number of metacentric chromosomes did not differ greatly between strains L-P55 and L-P59. They varied from sample to sample, but both maintained an average value of 13. Most metacentrics were very large and conspicuous, except for one which was small. In L-P55 all the large chromosomes were metacentrics, but in L-P59 the largest one was a submetacentric (figs. 1, 9, and 10). When fixation was favorable, many metacentric chromosomes had secondary constrictions, usually near one or both sides of the centromere (figs. 1 and 9). It could very well be that some of these chromosomes were dicentrics with the 2 centromeres close to each other. The subtelocentric chromosomes were all in the medium-small-sized category (figs. 1, 9, and 10).

Another interesting feature about the chromosomes of the L-P lines was the D chromosome. As reported in a previous paper (*10*), the D chromosome was a long, subtelocentric element with a deep secondary constriction in the long arm. It was at first regarded as a dicentric chromosome. The chromosome could be roughly divided into 3 sections: the short arm, the middle piece which was approximately twice as long as the short arm, and the end piece located at the other side of the secondary constriction. The end piece was slightly shorter than the short arm, and seemed to be somewhat heteropyknotic. In well-fixed cells the middle piece of the D chromosome could be subdivided into 3 sections by 2 less conspicuous secondary constrictions. The first section of the middle piece, located immediately below the centromere, was about as long as the short

arm. The other 2 segments were about equal in length, and each was approximately half as long as the first. In figures 9 and 11 the entire middle piece appears to be a single unit, but in figure 10 suggestions of additional secondary constrictions exist. In figures 5 and 12 the secondary constrictions are especially clear, giving the entire chromosome a ruffled outline.

Some cells in L-P55 had 2 such D chromosomes and some contained none. In the 1957 sample a mean value of 1.2 D chromosome per cell was recorded; in the 1958 sample the frequency dropped to 0.8. In L-P59 the January, 1959, sample studied by Dr. G. K. Manna showed a low value of 0.6 D chromosome per cell, but in the July sample the value had increased to 1.0. Of 50 cells randomly selected for analysis, 48 contained 1, one contained 2, and the remaining cell contained none.

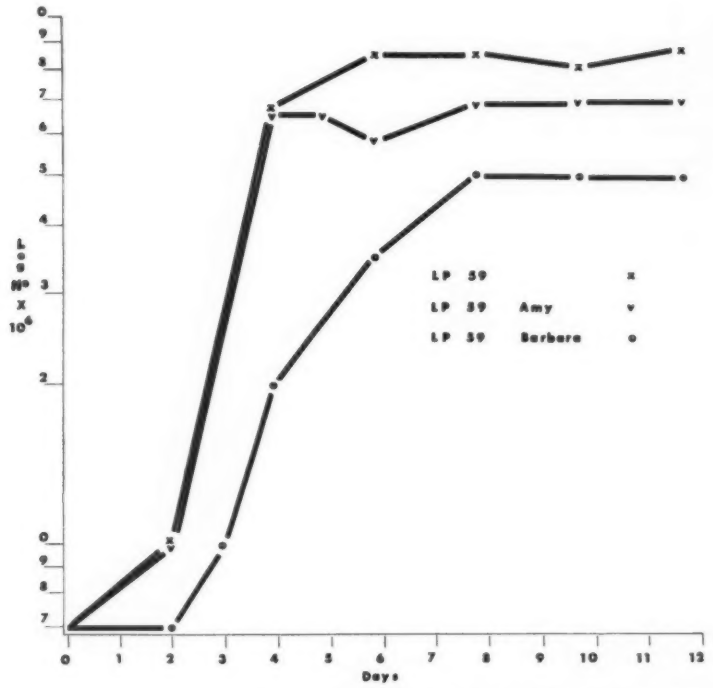
In the sample studied by Manna, just mentioned, when the D chromosome was absent, a very large metacentric chromosome sometimes appeared. This chromosome became one of the important markers in the strains described in this paper. For the sake of easy reference in the future, we prefer to use a name for each chromosome. This large metacentric is termed the "Victoria" chromosome. Actually, if the L-P populations are studied carefully, some unusual chromosome types can always be found, such as extremely long telocentrics, dicentrics, acentric fragments, or even ring chromosomes. Figure 2 shows an extremely long, multicentric chromosome. Minute chromosomes, which were not observed in strain L-P55, occurred in L-P59. Some of the minutes even had 2 distinct arms. All these aberrant chromosomes, however, never appeared consistently in the populations.

Strain L-P59 was a fast grower. In text-figure 1 the growth curve for L-P59 was an avarage taken from 4 sets of experiments. In 5 ml. of growth medium and with 5×10^5 cells as inoculum, the population reached between 6.25×10^6 and 8×10^6 cells in 6 days.

Strain Amy

Strain Amy was derived from a culture of L-P59 treated with colchicine for 3 weeks. There was a 1-week interval in regular medium between the 2d and the 3d weeks. The recovery took approximately 1 month. In strain Amy all cells were larger than those of the parental line except the polyploids. Figure 4 represents a field photographed from a preparation approximately 3 months after the removal of the final colchicine treatment. Notice the abundance of micronuclei and lobated nuclei, which indicate c-mitosis in the absence of colchicine. In figure 3, one such cell is presented in detail. In ciné-records, cells of strain Amy exhibited a high frequency of abnormal mitosis, especially of c-mitosis and multipolar spindles.

The first sample of strain Amy for cytological studies was taken approximately 1½ months after the removal of the final colchicine medium. In this population, nearly 95 percent of the cells were in the double-stemline



TEXT-FIGURE 1.—Growth curve (log scale) of L-P59, Amy and Barbara.

zone and the remainder was composed of higher polyploids of various caliber. None of the cells observed were the stemline type. In the second sample, taken 2 weeks later, the frequency of the 2s cells rose to 98 percent. Among the 2s cells the chromosome numbers varied from 118 to 138, almost the exact doubling of the s numbers, 59 to 68 (fig. 5). However, several cells showed odd chromosome numbers, 127, for instance, indicating that changes had already taken place after doubling. Moreover, idiogram analysis showed that not all the chromosomes could be paired. In both figures 11 and 12 the longest subtelocentric chromosome was single. In table 1 the mean values for the total chromosome number and the numbers of metacentrics, subtelocentrics, and D chromosomes are listed.

Two months later, a rough cytological check of strain Amy was made. No significant change in chromosome numbers was noted. Strain Amy was thus a double-stemline derivative of L-P59.

A growth curve (text-fig. 1) constructed from several experiments indicated that with a given amount of medium and a given amount of inoculum, the total cell number of Amy attained after 6 to 8 days of incubation was 4 to 5 $\times$ 10^6 cells—half the amount of the parental line under similar conditions. However, if one uses total cell volume, total chromosome numbers, or total amount of DNA as criterion, the values for L-P59

and Amy were almost the same. The average amount of DNA per 10^6 Amy cells was 44.7 μg. as compared with 25.9 μg. for L-P59.

Strain Barbara

Strain Barbara was originated in the same manner as strain Amy. Cell recovery of Barbara was noted at approximately the same time as for Amy, but the Barbara culture grew rather slowly at the initial stages. The population consisted of large, apparently polyploid cells as well as of small, spindle-shaped cells. The spindle cells appeared to be more elongated than the cells of the parental line.

In the first sample of strain Barbara, taken approximately 2 months after removal of the last colchicine treatment, it was immediately discovered that the population was not composed purely of double-stemline cells like strain Amy. A rough survey of the mitotic cells in this sample revealed that 54 percent of the population consisted of s, 42 percent of 2s, and 4 percent of higher polyploids.

Of special interest was the karyotype of the cells in strain Barbara. The characteristic D chromosome was missing entirely. Instead, a number of cells had some special chromosomes that were ordinarily not in the parental population. Figures 13 and 14 are camera-lucida drawings taken from various cells of the first sample of strain Barbara. These include extremely long subtelocentrics (figs. 13a and b), telocentrics with intercalary constrictions (fig. 13c), long subtelocentrics with one or more secondary constrictions (figs. 13d and e), dicentric chromosomes (figs. 13f through h), extremely long telocentrics (fig. 13i), and ring chromosomes (fig. 13j). In some of the double-stemline cells, these monstrous chromosomes were found to have doubled (figs. 14a through c), which indicated that the mitoses of these cells had been arrested once during the periods of colchicine treatment.

Although no critical counts were made with cells of the first sample, it was obvious that one cell type was the dominating genome of this population. The cells contained 59 to 61 chromosomes with no D chromosome. There were 13 to 15 metacentrics of various sizes and 2 or 3 subtelocentrics, one of which was considerably longer than usual. Most conspicuous was the extraordinarily long metacentric (the Victoria chromosome) occasionally observed in the parental line (figs. 15 through 17). Also common was a relatively long telocentric with 3 secondary constrictions (the Caterpillar chromosome). Although without evidence, the Caterpillar chromosome appeared to us as the long arm of the D chromosome (figs. 15 through 17). Morphologically, the Caterpillar chromosome resembles the ruffled chromosome reported by Rothfels *et al.* (*14*).

Another chromosome that was frequently observed from cells in the first sample of strain Barbara was a long telocentric with a deep constriction at the middle (the Wasp chromosome, figs. 16 and 17). It is not certain whether the middle constriction of the Wasp chromosome was also a primary constriction. In later generations of strain Barbara

this chromosome rapidly disappeared. In figure 6 the Victoria and the Wasp chromosomes are both clearly seen. The Caterpillar constrictions are visible but not clear (7 o'clock). In addition this cell had a ring chromosome.

In the second sample, taken approximately 2 weeks after the first one, cells with monstrous chromosomes had already dropped in number. The dominating type just described had become the representative genome or the stem line. In all these cells the Victoria and the Caterpillar chromosomes could be observed without difficulty; but cells containing the Wasp chromosome were lower in frequency than those in the first sample.

A count of 25 cells taken from the second sample (table 1) revealed that this strain had an average chromosome number of 60, four chromosomes below the value of the parental strain L-P59. The numbers of metacentrics and subtelocentrics per cell did not seem to differ significantly from the parental values.

The third sample of Barbara was analyzed 1 month after the second. By this time the strain was well established and its growth assay had been completed. The most significant change noted from this sample was the further reduction of the total chromosome number. The average chromosome number per cell had dropped from 60 to 58; but the number of metacentrics seemed to increase considerably (table 1). Its karyotypic variation was also appreciably greater than the previous population. In some cells the Victoria chromosome was twice represented; in others, either the Victoria or the Caterpillar was missing. The Wasp chromosome had nearly completely disappeared. Figure 18 is a complete idiogram of a cell from this population and shows 55 chromosomes in total. Among them were a Victoria and 2 long metacentrics only slightly shorter than the Victoria, one of which had a secondary constriction on one of the arms. The Caterpillar chromosome was present, but there was an especially long telocentric that also showed suggestions of 3 secondary constrictions.

The last sample of Barbara was taken 2 weeks after the third. The Victoria chromosome was no longer an indispensable feature of the cells. A number of the cells lacked both the Victoria and the Caterpillar chromosomes. There was a continuous decrease of total chromosome number per cell and an increase of the number of metacentrics (table 1). One cell of this sample contained as low as 49 chromosomes. From the parental line with 64 chromosomes among which 13 or 14 were metacentrics, strain Barbara gradually reduced its total chromosome number to 55, but increased its metacentrics to 18. A number of cells in this sample again showed new chromosomes, such as dicentrics and rings. These phenomena strongly suggested that the population was not yet stabilized. Table 2 lists the individual chromosome numbers counted from the 3 samples.

Strain Barbara was a slow grower. Results of growth experiments showed that the cells grew with the same inoculum and in the same amount of medium as the parental line, to between 2.5×10^6 and 3.1×10^6 cells,

TABLE 2.—Distribution of chromosome numbers in 3 samples of strain Barbara

Chromosome number	June 1959	July 1959	August 1959
49			1
50			3
51			
52			2
53			2
54	1	2	1
55		2	4
56		2	4
57	2	4	2
58		7	2
59	6	3	
60	6	1	3
61	3	5	1
62	5		
63	2		
Total	25	25	25

approximately one third the cell number attained by the parental strain (text-fig. 1). Furthermore, the population size did not increase any more in the original medium unless fresh medium was applied.

After the first sample of strain Barbara was taken, we had 2 stock bottles. One of them was saved for propagation and maintenance of the line, and the other was retreated with colchicine for another week. Later, this bottle was subcultured, one of which became subline Barbara-1. The other culture was again treated with colchicine for a week and subcultured in a similar manner to produce subline Barbara-2. Cytological values of these 2 sublines are also included in table 1.

In Barbara-1, monstrous chromosomes were once again abundant. The Wasp chromosome was observed frequently. In figure 7 a dicentric chromosome is seen, and in figure 8 there is a long telocentric with a secondary constriction. This chromosome was not the Wasp chromosome because it was much longer and the location of the constriction was not at the middle of the arm. Idiograms were made for 2 cells of Barbara-1 (figs. 19 and 20). The cell in figure 19 contained 18 metacentrics and 5 subtelocentrics. One of the metacentrics had one arm similar to the appearance of the Caterpillar chromosome which was missing in this cell. In the cell in figure 20 a ring-shaped dicentric chromosome was noted. This chromosome was actually not a ring chromosome. It was probably formed by fusion of 2 telocentrics at their free ends. In Barbara-2 populations, however, monstrous chromosomes were not exceedingly common. Both sublines, especially the latter, grew more slowly than the original line Barbara.

Strain Carolyn

When successful isolation of a double-stemline strain from L-P59 as well as isolation of a slow-growing strain with low chromosome number had been accomplished, it was considered important to analyze a population after a relatively short exposure to colchicine to detect whether during the

early stages of recovery there would be a shift of the population composition in favor of the less dominant genomes. A 1-day-old culture of L-P59 was treated with colchicine for 48 hours. Giant cells were, as expected, exceedingly common after the removal of colchicine medium. However, small cells quickly repopulated, and subcultures were made only 1 week after the termination of the treatment.

The first sample for cytological studies of this subline strain Carolyn, was taken 18 days after the removal of colchicine. The double- and multiple-stemline cells comprised only about 13 percent of the population.

Particular attention was given to the cells in the stemline zone. It was immediately noted that among the cells in the stemline zone, instead of over 90 percent, only 57 percent contained the characteristic D chromosome. However, a survey of 50 cells containing the D element revealed that the average chromosome number was reduced from 64 in the parental line to 62. Table 3 shows the distribution of chromosome numbers in the parental population and in populations of strain Carolyn. It is obvious that the representative genomes of the parental line, 64 to 66 chromosomes, were significantly lower in frequency than the lower chromosome numbers, 60 to 62.

Among the cells without the D chromosome, many contained the Victoria chromosome (figs. 21 and 23) and the Caterpillar chromosome (figs. 23 and 24), which were characteristic of strain Barbara. A new chromosome was also frequently observed, namely, a medium-sized subtelocentric with a deep secondary constriction near the end of the longer arm (figs. 21, 22, and 23).

Another interesting feature concerning the genomes without the D chromosome in the first sample of Carolyn was that the total chromosome number was much higher than in cells with the D chromosome. Cells with 66 to 68 chromosomes comprised nearly 80 percent of this subpopulation.

It was assumed that cells with 64 to 66 chromosomes including a D chromosome were the most vigorous genomes heavily set back by the colchicine treatment because of their great mitotic power. In order to detect whether these genomes could regain their dominance, strain Carolyn was subcultured twice a week. In table 3 data on the second sample, taken approximately 2 weeks after the first, and those of the third sample, 3 weeks after the second, clearly showed that the lost genomes had returned. Cells containing the D chromosome increased their frequency to 72 percent in the second sample and 90 percent in the third.

As an additional check, another culture of L-P59 was treated with colchicine in a manner similar to that used to derive strain Carolyn. Two weeks after the removal of colchicine, the population was examined cytologically. Cells containing the D chromosome dropped from the original frequency, over 90 percent, to 73 percent. However, no detailed analysis was made and the culture was discarded.

TABLE 3.—Distribution of chromosome numbers in 3 samples of strain Carolyn, with the parental population L-P59 for comparison

Population		Subpopulation	Chromosome number: 55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	Number of cells
Strain Carolyn	First sample	With D	1	1	1	3	4	6	3	11	4	3	2	8	1	2		50
		Without D							1		2	2	4	16	10	13	2	50
	Second sample	With D						1	1	1	8	4	6	2		2		25
		Without D						2	2		1	3	5	7	3	2		25
	Third sample							3	2	5	2	7	3	2				25
Strain L-P59						1	1	4	5	5	5	7	8	8	4	2		50

Additional Data

Twelve more lines have been established from L-P59 following long-term colchicine treatment. In no case was there a complete conversion to double stem line as in strain Amy. In 8 lines so far studied cytologically, the D chromosome was present at a low frequency.

Growth assays made for 4 lines proved that they were all slow growers. With 5×10^5 cells as inoculum and 5 ml. of growth medium, the final cell counts after 6 days of incubation are shown below for 4 cell lines:

Strain	Final cell count ($\times 10^6$)
Dorothy	1. 6
Edna	1. 0
Fay	2. 1
Gloria	1. 3

It seems that, except for strain Fay, all lines grew even more slowly than Barbara.

DISCUSSION

The production of strain Amy demonstrated that it is possible to produce double-stemline strains without even using the cloning method. From the growth experiments we can easily see that strain Amy was actually as efficient as the parental line, if we consider synthesis of total cellular material on a per DNA or on a per chromosome-set basis. Our results were very similar to those obtained by Hauschka *et al.* (*15*) on growth *in vivo* of diploid and tetraploid mouse tumors. However, in a growing cell population, cellular multiplication counts more than any other criterion in the struggle for survival, so that with equal efficiency the double-stemline cells are handicapped. The fact that they are not entirely eliminated from the populations may be due to two reasons: (1) they are constantly generated by the stem cells through various polyploidization processes, and (2) they may have some metabolic advantages under certain environmental conditions which, perhaps, is a part of the normal culture cycle. We shall elaborate this point in the following paragraphs.

In cell populations where cell growth and cell proliferation, instead of maturation and differentiation, form the primary objective, genomes that assimilate nutrients fastest and enter mitosis most frequently will eventually become the dominating types. Let us assume that the genomes in a cell population can be classified into several grades according to their growth rate and their reproductive capacities. When a population is subcultured in new culture vessels, the vigorous genomes would probably be the first group of cells to resume growth and enter mitosis. They are followed by the second-rate and third-rate genomes. By the time the most inferior genomes enter their first cell division, the superior ones may have already divided several times. Thus it is not unreasonable to deduce that frequent subculturing would accelerate the overproduction of the stem cells, whereas permitting the populations to remain in the stationary

growth phase would conserve the existing mutants. On the other hand, when a population in the log growth period is subjected to the effect of colchicine, the genomes that would be poisoned most severely should be the stem cells.

If the foregoing supposition is reasonable, we can describe the genetic composition of strain L-P59 based on the results obtained from the present study. In the first sample of strain Carolyn, which was derived from a growing population treated with colchicine for only 48 hours, the genomes greatly reduced in number were the most common types in strain L-P59, namely, cells with 63 to 66 chromosomes including a D element. These genomes regained their dominance in the second and, especially, the third samples after diligent subculturing, without allowing the populations to go into a stationary stage. The second-rate genomes seemed to be of two types: (1) cells with 58 to 62 chromosomes including the D chromosome, and (2) cells with 66 to 68 chromosomes but without the D. These were the most common cell types recorded in the first sample of strain Carolyn.

It is rather obvious that cells with chromosome number lower than 60 were more inferior, as evidenced by the cytological characteristics of, and the growth experiments made with, cells of strain Barbara. Apparently a number of genomes remained after the aggressive types had been removed by the long-term colchicine treatment. A number of the recovering genomes may have been formed by the abnormal mitoses of the double or multiple stem cells. Among these genomes, which were all metabolically inferior when compared with the stem cells or even with the second-rate genomes, competition again started. In the absence of the stem cells, those with a slight metabolic advantage would theoretically gain dominance and gradually crowd out the others. However, the situation was not so simple. It seemed that none of the surviving genomes of strain Barbara was really suitable for continuous propagation, so that a period of genotypic reshuffling, manifested by readjustments of chromosome number as well as structure, was observed.

In this connection, it should be mentioned that the abundance of chromosome monstrosities found in strain Barbara was a result of selection rather than inducement because there is as yet no conclusive evidence to show that colchicine causes chromosomal breakages (*16*) and because abnormalities can occasionally be found in the parental population.

As we have discussed, generous subculturing may help the stem cells to overpower other genomes. If, on the other hand, the cultures were always permitted to remain at their stationary phase for as long as possible, other genomes might be more adaptive than the stem line. Therefore, "stem line" is a relative term, depending upon the conditions of the environment. Explicitly speaking, in a cell population there may be several optimum classes of genomes, each being suitable for one type of environment, and an equilibrium is reached according to the environment provided for them. We may recall the change of chromosomes in L-P55 which later became L-P59. The reserve bottles, because they were spare, were naturally not

propagated vigorously. Most of the time the cultures were in their stationary growth phase. When the stocks were first propagated, the frequency of the D chromosome was only 0.6 per cell (table 1). After repeated propagation, the frequency went up to 1.0. This suggests that cells containing the D chromosome and those containing no D differed in their metabolic optima. What we term superior or inferior is only in regard to one aspect, namely, continuous multiplication.

We may consider, according to the foregoing hypothesis on superiority, that strain Barbara was not inferior if the cultures were kept constantly in their growth plateau. Since we tried to propagate the cells for experimental purposes and the genomes were not so suitable for such an environment, evolution started again. Perhaps cells with 60 ± chromosomes appearing during the early period had some undesirable chromosomes for perpetual growth, so that readjustment was required. The readjustment could possibly be performed by either gaining more desirable genes so as to compensate for the harmful ones or by eliminating the undesirable chromosomes or parts of chromosomes. Actually the latter was what happened. It must be pointed out, however, that the reduction of chromosome number in strain Barbara was not as drastic as indicated in table 2, if we consider also the increase of the metacentrics which consisted of two arms each. Just how the population of strain Barbara may eventually stabilize remains to be seen.

By the same token, the double-stemline cells may also be advantageous genomes for a certain phase of the culture cycle. This is perhaps one of the reasons that they were never completely out of the picture just as the "inferior" genomes were never completely lost.

There may be a strong similarity between the population structures of transplantable tumors and cell strains *in vitro*. If transplantation of a tumor is always made at the last minute of the host's life, the resulting population may well be different from that transferred always at the peak of cellular growth. It is possible that if Kaziwara repeated his stresses to the Ehrlich tumor populations every time he made a transfer, the populations might completely shift to the 2s direction. On the other hand, the "stem cells" that recovered after the stress given by Makino and his associates may have possessed many subtle genetic alterations not readily detectable. Since there are so many internal and external factors in the development and maintenance of cell populations, it is perhaps more appropriate to view the populations as dynamic entities which are delicately balanced and are extremely flexible.

The present study suggests a method by which either double-stemline strains can be induced or metabolically inferior mutants can be selected from cell populations *in vitro*. It is of great interest to cytologists that there seemed to be some relationship between chromosome constitution and growth capacity of mammalian cells. In view of the fact that strain Barbara grew reasonably well during the first few days of subculture but leveled off the growth curve much sooner than did the parental line with the same amount of nutrient medium, there is a possibility that some sub-

stance or substances acted as limiting factors. Nutritional studies on strain Barbara and other slow-growing strains now being established may reveal information on the possible role of certain chromosomes in metabolic processes.

The colchicine method described here should be applicable to other cell strains *in vitro*. If cloning techniques can be employed on populations previously treated with colchicine, one should be able to isolate many mutant genomes in relatively pure form.

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PLATES

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PLATE 93

FIGURE 1.—A cell from strain L-P59: 60 chromosomes. *Outline arrow* points at the D chromosome; *solid arrow*, at a metacentric with a secondary constriction near the centromere. × 1,800

FIGURE 2.—A cell from strain L-P59 showing an unusually large chromosome with many constrictions. Abnormal genomes are often found in the populations, but seldom are they identical. × 1,800

FIGURE 3.—A cell from strain Amy showing multiple micronuclei. Such cell suggests that it had a spindle failure during the preceding mitosis. × 1,800

FIGURE 4.—A low-magnification field showing cells of strain Amy. Three double-stemline metaphases, many c telophases and interphases. × 700

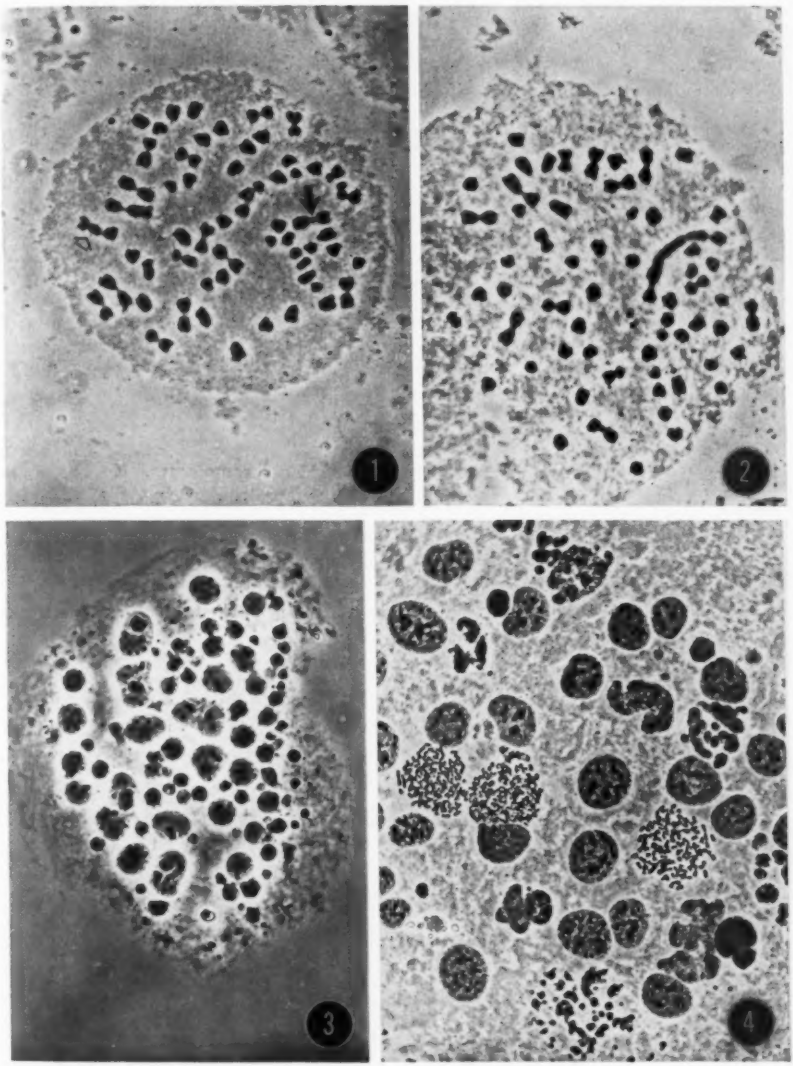

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PLATE 94

FIGURE 5.—A cell from strain Amy showing 126 chromosomes. *Outline arrows* point at the D chromosomes. *Note* secondary constrictions in the middle piece. × 1,800

FIGURE 6.—A cell from strain Barbara showing 54 chromosomes with a ring chromosome (short, *solid arrow*), the Wasp chromosome (long, *solid arrow*), and the Victoria chromosome (*outline arrow*). The constrictions of the Caterpillar chromosome (7 o'clock) are not especially clear. × 1,800

FIGURE 7.—A cell from strain Barbara-1 showing 55 chromosomes with the Victoria chromosome (*outline arrow*) and a dicentric element (short, *solid arrow*). The long, *solid arrow* points at a chromosome with secondary constriction near the centromere. × 1,800

FIGURE 8.—A cell from strain Barbara-1 showing 54 chromosomes with the Victoria chromosome (*outline arrow*) and a long telocentric which has another constriction near the free end (long, *solid arrow*). This chromosome is not the Wasp chromosome which has the second constriction in the middle of the long arm (*cf.* fig. 6). × 1,800

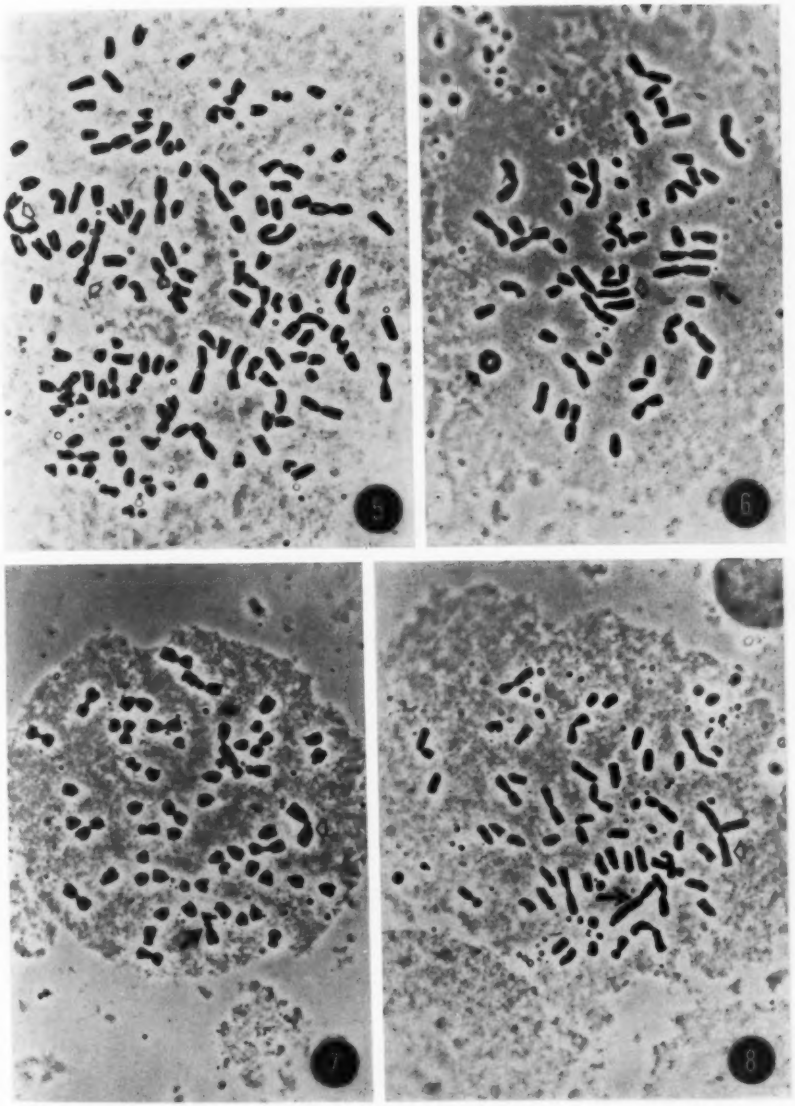

5
6
7
8

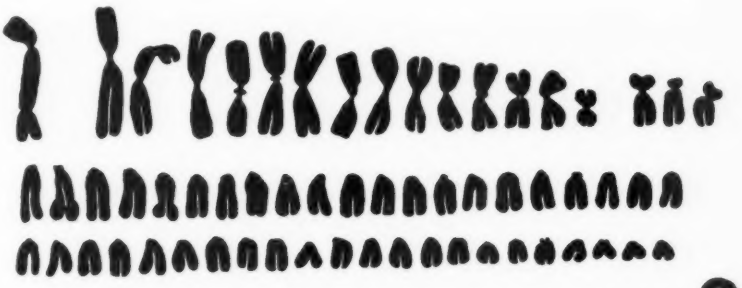
PLATE 95

FIGURE 9.—A complete idiogram of a cell from strain L-P59: 61 chromosomes.

FIGURE 10.—Same as figure 9: 64 chromosomes.

FIGURE 11.—Idiogram of a cell from strain Amy. Telocentrics excluded.

FIGURE 12.—Same as above. *Note* secondary constrictions in the middle piece of the D chromosomes.



9

10

11

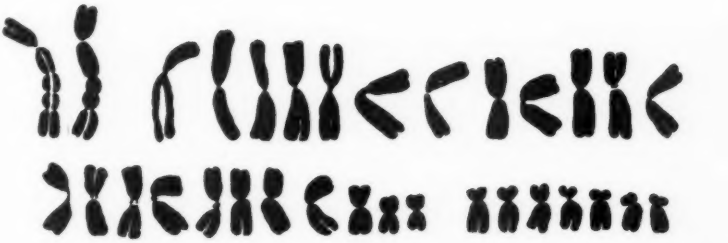


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PLATE 96

FIGURE 13.—Some monstrous chromosomes collected from various cells in the first sample of strain Barbara immediately after the population had recovered from the long-term colchicine treatment.

FIGURE 14.—Monstrous chromosomes in pairs from cells in the double-stemline zone. Same sample as in figure 13.

FIGURES 15 to 17.—Idiograms of 3 cells from strain Barbara showing metacentrics, subtelocentrics, and marker chromosomes only. The Victoria chromosome is arranged at extreme *left* of each row.

FIGURE 18.—A complete idiogram of a cell from strain Barbara: 55 chromosomes.

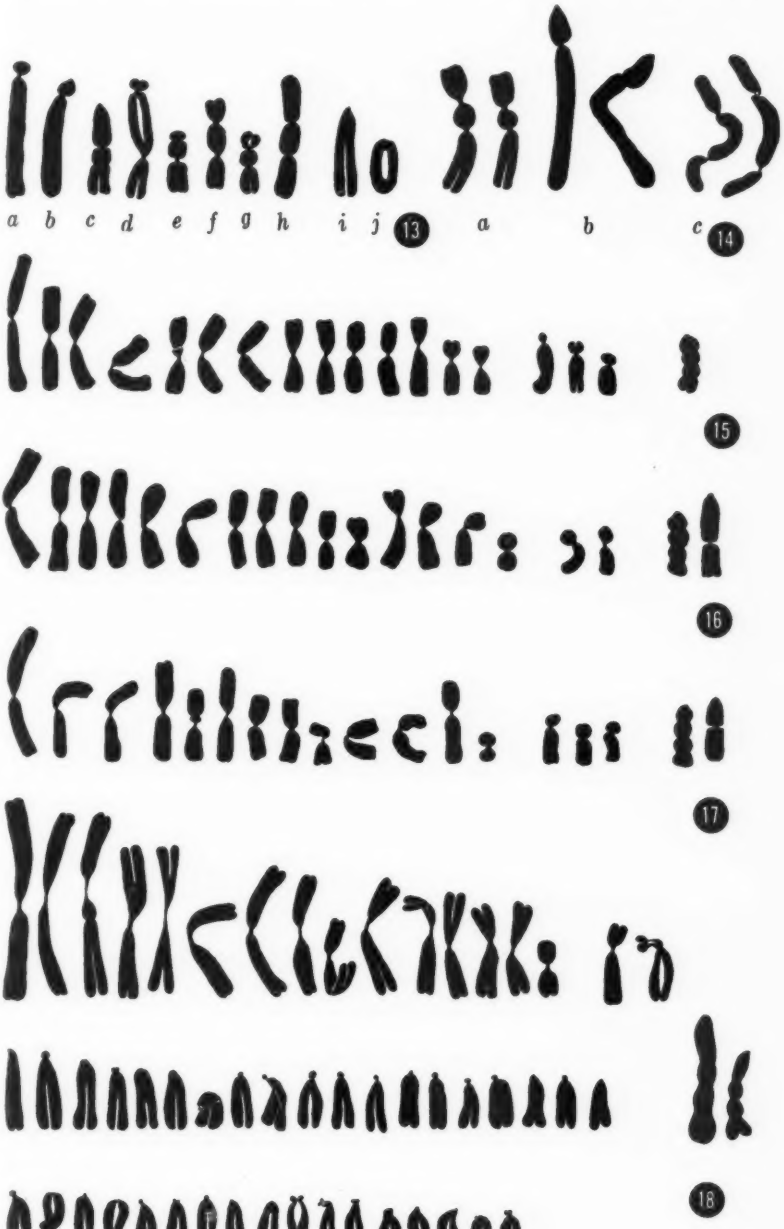
a b c d e f g h i j 13 a b c 14
15
16
17
18

PLATE 97

FIGURES 19 and 20.—Idiograms of 2 cells from strain Barbara-1, showing metacentrics, subtelocentrics, and marker chromosomes only. *Note:* one arm of the third chromosome from *left* of figure 19 resembles the Caterpillar.

FIGURES 21 to 24.—Idiograms of 4 cells from strain Carolyn, showing metacentrics, subtelocentrics, and marker chromosomes only. *Note* subtelocentric with a secondary constriction in figures 21 through 23.

19
20
21
22
23
24

Induction of Tumors in Syrian Hamsters by a Cytopathogenic Virus Derived From a C3H Mouse Mammary Tumor [1, 2]

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SUMMARY

Tumors of the kidney and heart have been induced, by a cytopathogenic virus, in Syrian hamsters. The virus, which had been recovered in tissue culture from an extract of a C3H/Jax mouse mammary tumor and serially cultivated *in vitro* on normal mouse cells, was injected subcutaneously into the hamsters on their 1st day of life. In 3 experiments with both random-bred and partly inbred hamsters, over 90 percent of animals died with tumors. Mortality was highest around the 12th day after the animals received the virus, and at autopsy, in most of the animals, both kidneys were massively enlarged and diffusely infiltrated with tumor. A second, lower peak incidence of death occurred around the 19th day; in these animals, nodular tumors of the kidney and heart were found. The tumors were spindle-cell sarcomas which were locally invasive and many metastasized to the lungs. A number of these tumors grew progressively after transplantation into adult hamsters and killed their new hosts. Hemorrhage into the peritoneal and pleural cavities was common in animals that died with extensive tumors after receiving the virus. Localized vascular dilatations were also commonly found in the liver, lungs, intestine, and reproductive organs of these animals. At the surface of the liver and lungs, the dilatations formed blood-filled blebs, and rupture of these blebs probably led to the hemorrhages that were observed. Newborn hamster controls that received supernatant fluid from cultures of normal mouse kidney cells, normal monkey kidney cells, or fresh culture medium developed no tumors during the first 10 weeks thereafter. A number of animals that received fluid from normal mouse kidney cultures were retained for 5½ months and they remained free of tumors. However subcutaneous tumors developed in a number of animals that received fluid from monkey kidney cultures and in one that had received fresh medium only, after a long latent period, which indicated that these animals had somehow received a small dose of virus. The results demonstrate an extraordinarily rapid and regular induction of malignancy by a mammalian tumor virus.—J. Nat. Cancer Inst. 24: 1095–1111, 1960.

IN A previous communication (*1*) we described a cytopathogenic virus that had been recovered from a C3H mouse mammary tumor and induced tumors when injected into newborn Swiss mice. This report included

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[2] This investigation has been supported by a grant from the National Cancer Institute of Canada.

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preliminary results showing that the virus also induced tumors in hamsters, with death sometimes occurring as early as 10 days after injection.

The purpose of the present paper is to describe in detail the results of giving the virus to newborn hamsters; it includes data on the incidence, latent interval, pathology, and transplantability of the tumors, and a description of the hemorrhagic lesions that were observed.

The relationship of the virus used in the present experiments to other tumor viruses, particularly the polyoma virus, based on its properties *in vivo* and *in vitro*, including its appearance under the electron microscope, will be described and discussed in a separate report (*2*).

MATERIALS AND METHODS

Randombred Syrian hamsters (*Mesocricetus auratus*) were used, except in one experiment in which the hamsters had been inbred to the extent that they were the result of brother × sister mating for 7 generations. Pregnant females near term were obtained from a commercial breeder, High Oak Ranch, Richmond Hill, Ontario. They were kept on straw and sawdust beds in metal cages. Lettuce, carrots, spinach, and Ralston Purina Mills Laboratory Mouse Diet pellets were fed *ad libitum*. Drinking water was not supplied.

The virus was prepared according to the procedure already described (*1*). Briefly, a spontaneous mammary tumor from a C3H/Jax retired breeder female served as source material; the tumor was homogenized and gently centrifuged, and the supernatant fluid was used to infect a fresh culture of normal C3Hf/HeHa mouse kidney cells in medium CMRL–1066 with 20 percent horse serum. This culture was destroyed by the 13th day and fluid from it was used to infect a fresh culture. Cytopathogenic effects were then observed after 4 days and further consecutive passages were made every 3 to 6 days. The virus preparations for the present experiments were pools consisting of supernatant fluids from cultures of the 14th to the 16th passage, frozen at −10° C. These fluids caused agglutination of guinea-pig red cells with titers ranging between 1:320 and 1:1280.

Hamsters were injected with virus during the 1st day of postnatal life. The virus preparations were thawed and 0.2 ml. of the fluid was injected under the skin of the back.

In early experiments, young animals were lost because they were eaten by their mothers, and it was assumed that many of these were eaten alive. When the animals were checked more frequently, however, it was found that some of them had died before being eaten and that death was the result of tumor, hemorrhage, or both. Routinely, therefore, the animals were checked twice daily and those found dead were autopsied. In addition, listless animals were examined and considered moribund if their kidneys could be palpated as large hard masses, or if by moistening the abdominal skin, intraperitoneal hemorrhage could be seen. These

moribund animals were killed; in the results they are considered separately from those found dead.

For histological examination, organs were fixed in 10 percent formalin, embedded in paraffin, sectioned at 4 mμ, and stained with hematoxylin and eosin. In addition a number of kidney and heart sections were stained by Masson's trichrome method. Others were prepared by the periodic acid-Schiff method and counterstained with hematoxylin.

For tumor transplantation, whole kidneys or hearts containing tumor were chopped finely, with scissors, in Krebs-Ringer-phosphate solution (*3*) with added gelatin (2.5%), penicillin (100 units/ml.), and streptomycin (100 μg./ml.). The chopped tissue of one or two whole hearts or kidneys was injected subcutaneously or intraperitoneally into randombred adult hamsters. Tumors that arose in partly inbred hamsters were similarly transplanted into animals of the same generation.

EXPERIMENTS

The effects of injecting the virus into newborn Syrian hamsters were studied in 3 experiments. In the first, 163 randombred hamsters received virus that was serially cultivated on normal C3Hf/He mouse kidney cells. In the second, 40 randombred animals were given virus that had been carried for 3 passages on similar cells, which had received 5000 r of X rays. (This was done in connection with another experiment designed to determine whether the virus could be propagated on irradiated cells.) In a third experiment, 27 partly inbred Syrian hamsters received virus cultivated on normal Swiss ICR mouse embryo cells.

Three groups of newborn randombred Syrian hamsters served as controls. One group of 58 animals received supernatant fluid from normal C3Hf/He mouse kidney cultures; a second group of 50 animals was given supernatant fluid from normal monkey kidney cultures; and a third group of 18 animals received fresh medium CMRL–1066 with 20 percent horse serum. All controls were kept in a separate room from the one containing virus-injected animals, but both rooms were on the same floor.

Eight kidney tumors and 3 heart tumors that arose in the hamsters given virus when newborn were transplanted into adult hamsters. At least 2 recipients were used for each tumor.

RESULTS

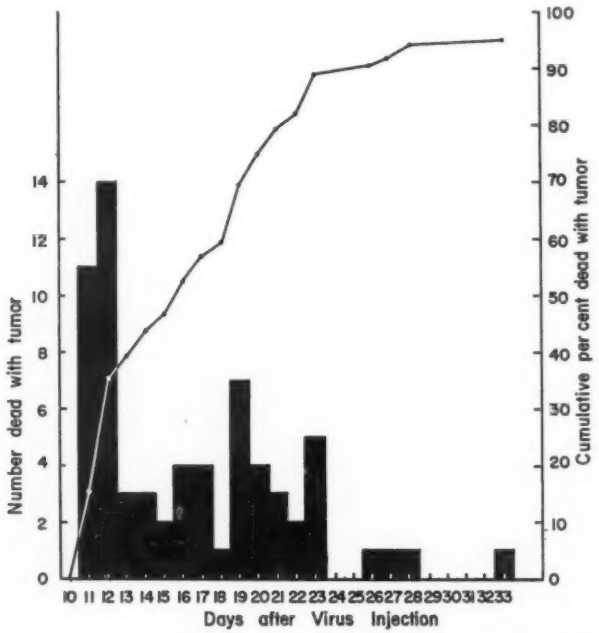
Tumor Induction

Experiment 1.—The number of noninbred hamsters that died within 33 days after receiving virus is shown in table 1. Most of these animals had massive intraperitoneal hemorrhage. All but 2 of the 69 animals

that died, with or without hemorrhage, had kidney tumors. Text-figure 1 shows the number and the cumulative percentage of animals that died with kidney tumor at various times after virus injection. The histogram shows that the peak mortality with kidney tumor occurred around the 12th day and a second, lower peak occurred at around 3 weeks. The median latent interval until death with kidney tumor was 16 days. Of 25 moribund animals killed during the first 33 days, 22 had kidney tumors. Two animals survived beyond 33 days; one of them, killed at 81 days, had a kidney tumor that was found only on microscopic examination and the other was normal.

TABLE 1.—Incidence of kidney tumors in Syrian hamsters receiving injection of virus cultivated on normal C3Hf/He mouse kidney cells, on 1st day of life

Hamsters injected: 163			
Data available on: 96			
Number found dead	69	Number killed	27
With tumor	67	With tumor	23
Number survived	2	Percent with tumor	85%
Percent dead with tumor	94%		



TEXT-FIGURE 1.—Mortality of Syrian hamsters with kidney tumor after they had received injection, on 1st day of life, of virus cultivated on normal mouse cells. Ordinate at *left* shows daily mortality of animals with tumor; ordinate at *right* shows cumulative daily mortality expressed as percentage of total deaths of animals with tumor.

Many animals that died or were killed had heart as well as kidney tumors. These tumors were not prominent in hamsters that were autopsied earlier than 16 days, although on microscopic examination tumors were found in many of these animals. Tumors grossly visible were found in the hearts of animals dying after the 16th day. Of a total of 82 hearts examined microscopically, from animals that died or were killed, 50 or 61 percent showed definite tumors.

Experiment 2.—Of 40 randombred hamsters injected with virus grown on irradiated mouse kidney cells, 15 were eaten, hence information is available on only 25. All these animals died 12 to 37 days after receiving the virus. All had kidney tumors and all but 2 also had heart tumors.

Experiment 3.—Of 27 partly inbred hamsters receiving an injection of the virus on their 1st day of life, information is available on 12. The rest were eaten. Three of these 12 were found dead on the 10th day after receiving an injection and at autopsy all 3 had tumors. Eight animals were killed on the 10th day and all but 1 had tumors. Only 1 animal survived beyond the 10th day and that animal died with bilateral kidney tumors and heart tumors on the 21st day.

Pathology

The appearance of the organs in virus-injected animals from all 3 experiments was similar, both grossly and microscopically. Therefore the following description will serve for all.

The morphology of the tumors in the kidney and heart was different in animals that died early, up to 16 days after virus injection, from that in animals that died late, during the latter part of the 3d week and thereafter. The kidneys of animals dying early were usually massively enlarged, always bilaterally, and often 10 or more times their normal size. Such kidneys, however, always retained their normal shape (fig. 1). Their cut surface (fig. 2) showed that the cortex and medulla were almost completely replaced by diffusely infiltrating tumor. In animals that died late the typical tumor was nodular; single or multiple circumscribed masses of solid tumor were present within the substance of the organ, either unilaterally or bilaterally, leaving the rest of the kidney uninvolved (fig. 4).

Microscopically the diffuse tumors seen in animals that died up to 16 days after virus injection consisted of infiltrating masses of spindle-shaped cells (fig. 3). The cells were usually disposed in whorls and bundles, with their long oval nuclei parallel to one another. The cells appeared to infiltrate between and distort tubules rather than invade them. Intercellular substance staining with Masson trichrome and with the periodic acid-Schiff technique separated the cells from one another.

In animals that died after 16 days, nodular instead of diffuse kidney tumors were the rule (fig. 4). Single tumors were rare; as many as 20 tumors were seen in a single microscopic section of the kidney (fig. 6) and they varied greatly in size. Large foci often were confluent. The

nodular tumors consisted of actively growing cells disposed in whorls and bundles and otherwise identical in appearance with cells seen in the early tumors (fig. 5). These nodules compressed and invaded the tissue immediately surrounding them. Necrosis and hemorrhage were often found in both diffuse and nodular kidney tumors.

Most of the hearts of animals that died 16 days or less after receiving virus appeared grossly normal and some had grayish flecks on their surfaces. The microscopic appearance of these hearts varied. Many of those that had appeared grossly normal had areas in which there were diffuse lesions of the interstitial tissue of the myocardium and necrosis of muscle cells. In such areas the interstitial spaces were wider than usual. Many of the cells in the spaces had swollen nuclei and in some of the latter the chromatin was marginated. In other hearts, only relatively localized patches of cells were seen; such patches were disposed beneath the epi- and endocardium or scattered in the substance of the heart muscle. While some had the characteristics described, others consisted of aggregations of spindle-shaped tumor cells arranged in whorls and bundles similar to those already described in the kidney.

In the hearts of animals that died after 16 days, discrete single or multiple solid tumor masses were seen growing out of the surfaces of the heart (fig. 7) or within the substance of the organ (fig. 8). The walls of one or more of the chambers were affected. These tumors occasionally were massive, filling a large part of the thoracic cavity. Microscopically the tumors were spindle-cell sarcomas (fig. 9). Except for sites of compression and invasion caused by tumors, the heart tissue appeared normal.

In addition to the kidney and heart tumors already described, small tumors were also occasionally seen on the serosal surface of the stomach and intestine. Microscopically these were also found to be made up of spindle cells and to be localized in the lamina propria of these organs.

The livers of the virus-injected animals regularly showed round spaces filled with blood throughout the thickness of the organ. Many of these blood-filled cavities protruded above the surface of the organ, forming blebs under the liver capsule (fig. 10). They varied from microscopic in size to over one-half centimeter in diameter. Microscopically they were seen to be discrete vascular dilatations lined by a more or less complete layer of endothelium and containing, in addition to blood, fragments of parenchyma and occasionally thrombi. Often their walls were ruptured and blood was found in the parenchyma around them. The hemorrhages into the peritoneal cavity which commonly occurred, probably resulted from the rupture of the exterior walls of blebs of this type. The livers also evidenced fatty change in and necrosis of parenchymal cells. This occurred most often in the midzone of the lobule and around the central vein; the periportal areas appeared unaffected. A small number of vascular dilatations in the livers of a few animals showed evidence of proliferation of cells in or associated with their walls. These areas of proliferation resembled the lesions observed in the hearts of animals given virus.

Vascular dilatations were frequently observed on the cecum, stomach, and reproductive organs. They were also seen on the surface of the kidneys massively infiltrated with tumor.

Three kinds of lesions were seen in the lungs of animals that died after receiving virus: hemorrhage, necrosis, and secondary tumor. The hemorrhagic lesions consisted of blood-filled cavities lined by endothelium. These lesions, similar to those described in the liver, commonly protruded from the surface of the lung and were covered by pleura (figs. 7 and 11). Localized hemorrhage into the lung tissue and hemorrhage into the pleural cavity probably resulted from rupture of these dilated vessels. Generalized dilatation of vessels with interstitial edema and hemorrhage were also frequent in the lungs. Cellular necrosis was manifested by the presence of patches of cells or scattered individual cells with pyknotic nuclei. Tumor masses were also observed; these were multiple, small, round, and mainly subpleural nodules, and appeared to be metastases from either the kidney or the heart tumors (fig. 12).

Tumor Transplantation

Of 8 kidney tumors transplanted into adult hamsters, 5 grew progressively in and killed their hosts in 3 to 10 weeks. One of 3 heart tumors transplanted into adult hamsters killed its host in 15 weeks. Some tumors remained localized and grew to enormous size. Others invaded and metastasized in their new hosts. Tumors arising in noninbred hamsters grew progressively in noninbred hamster adults. The numbers are too small, however, to permit comparison between the transplantability of these tumors and that of tumors from partly inbred hamsters in partly inbred hosts.

Controls

Among the 126 control animals, there was the usual small mortality (18 animals) in the first few days of postnatal life. Forty-nine animals that received fluid from normal mouse kidney cultures, 41 that received fluid from normal monkey kidney cultures, and 18 that received fresh medium survived the next 10 weeks and none developed tumors or hemorrhagic lesions within this period.

A small number of animals from each of the 3 control groups was retained for 5½ months, after which all were killed. In the first group which received normal mouse kidney culture fluid, of the 15 animals kept for this period, none developed tumors. Twenty of 23 hamsters in the second control group, which received normal monkey kidney culture supernatant fluid, developed tumors; this represents 87 percent of the animals that survived 5 months. The tumors were all subcutaneous and consisted of single or multiple solid, circumscribed masses either free in the subcutaneous tissue or attached to skin but not to underlying tissue (fig. 13). Microscopically these tumors consisted of spindle-shaped cells

with large amounts of intercellular substance. Multinucleated giant cells were frequent. There was no evidence of these tumors invading or metastasizing. The tumors were first detected at 4½ months and were slow-growing. Some reached massive size, and 2 of the animals bearing them died at 5 months. No kidney or heart tumors and no hemorrhagic lesions were detected in any of these animals. In the third control group, which was given fresh culture medium, 1 of 6 survivors developed a similar subcutaneous tumor.

DISCUSSION

Tumors of the kidney and heart induced in hamsters by our virus preparations invade locally, frequently metastasize, and kill the animals in which they arise. Many of them grow progressively on transplantation and result in the death of the new host. Therefore they satisfy the criteria of malignancy.

Histologically these tumors appear to be similar to those induced in hamsters by the polyoma virus, as reported by Eddy *et al.* (*4*). There are, however, three points of difference between their findings and ours, which deserve comment:

1. Tumors developed very rapidly in the kidneys of many of our animals and were massive and diffuse. Eddy *et al.* obtained kidney tumors after a longer latent period and these appear to have been only nodular. As described in the results of the present paper, nodular kidney tumors also developed in about a half of our injected animals, but all of these tumors occurred late, at a time when the animals that developed diffuse tumors were already dead. Preliminary results from a study now in progress show that hamsters receiving small quantities of the virus develop only nodular tumors late, while those receiving large quantities develop diffuse tumors after a short time. Since all animals in the present experiment were given the same dose of virus, but about a half of them developed diffuse tumors early and a half discrete tumors later, conditions determining the response to the virus must have differed among the animals. Thus one reason for differences in the latent period for the induction of kidney tumors and for differences in gross morphology of these tumors might be a difference in susceptibility of the injected animals. This could explain the discrepancy between the results of Eddy *et al.* and ourselves if it is assumed that the behavior of their virus and ours is identical. Or, making the same assumption, the quantity of virus given to our animals could have been higher than that given to theirs. Another possibility is that our virus is a variant of the polyoma virus and thus could have behaved differently in the animals.

2. Eddy *et al.* state in their report that they were unable to transplant their tumors, whereas a number of ours were transplantable. However,

transplantation of tumors in noninbred animals is haphazard and we understand that hamster tumors induced by polyoma virus have since been successfully transplanted. It is of course still possible that some of the tumors induced with our virus preparations were actually more malignant than theirs.

3. Widespread hemorrhagic phenomena were frequently observed in our experimental animals. Discrete dilatations of vessels were common in both the liver and lungs and clear-cut evidence of dilated vessels having ruptured was often seen. It might be questioned, in view of Eddy *et al.* having reported the finding of cavernous hemangiomas and hemangioendotheliomas in their injected hamsters, whether the dilated vessels that we observed were in fact tumors of this type. Whereas it is entirely possible that there were cavernous hemangiomas and hemangioendotheliomas that we did not identify in our animals, there is no question about the fact that the vast majority of dilated and ruptured vessels that we observed gave no histological evidence of being tumors. However, no attempts were made to transplant these lesions. The hemorrhagic condition found in our experimental animals was in some ways similar to the hemorrhagic disease produced in chicks by large doses of Rous sarcoma virus (*5*, *6*). The mechanism of production of this condition in our animals is unknown, though it is possibly the result of direct action of the virus on the developing blood vessels. Furthermore heart lesions due to the virus might have contributed to the dilatations and hemorrhages by causing increased pressure in the venous system. The small number of proliferative lesions associated with the walls of blood vessels in the livers of our injected animals resembled the nodules that we observed frequently in the heart; these proliferative lesions could thus have been primary mesenchymal cell tumors.

The development of a high incidence of subcutaneous tumors in the control hamsters that received injection, on their 1st day of life, of the supernatant fluid from normal cultures of monkey kidney cells requires some comment. In unpublished experiments we have found that giving newborn hamsters very small doses of the virus subcutaneously results in their developing subcutaneous tumors after a long latent period. Accordingly there is reason to believe that these control animals may have received a small dose of virus. The source of this virus is unknown, but it should be emphasized that it is very difficult to eliminate the possibility of contamination in a laboratory in which many animals are infected with virus.

The finding that we regard as being of particular significance in this work is the extraordinarily rapid induction of massive kidney sarcomas, which led to the death of a large number of our animals at around the 12-day period (text-fig. 1). The fact that these tumors developed so regularly and so rapidly suggested the feasibility of making the day-to-day study of the process of viral carcinogenesis that is reported in the following paper.

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PLATES

PLATE 98

FIGURE 1.—Hamster that received injection of virus on 1st day of life and was dead at 10 days of age. *Note* massive diffuse enlargement of both kidneys. × 2

FIGURE 2.—Cross section through kidney of animal in figure 1, showing typical diffuse infiltration of renal cortex and medulla with tumor. × 4

FIGURE 3.—Photomicrograph of hamster kidney showing cortex infiltrated with tumor. × 200

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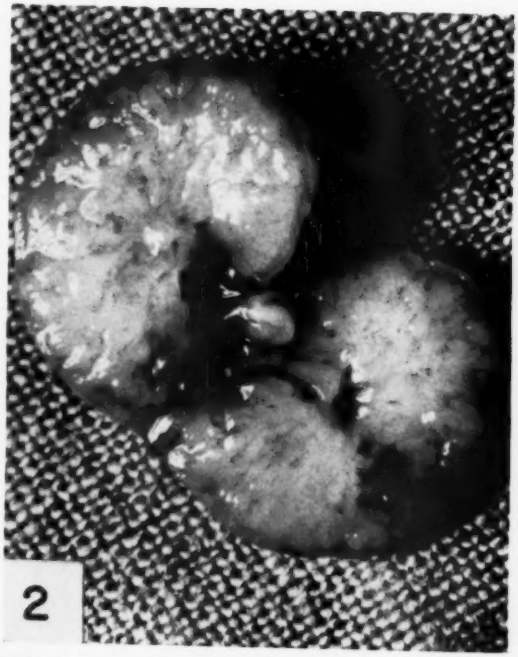

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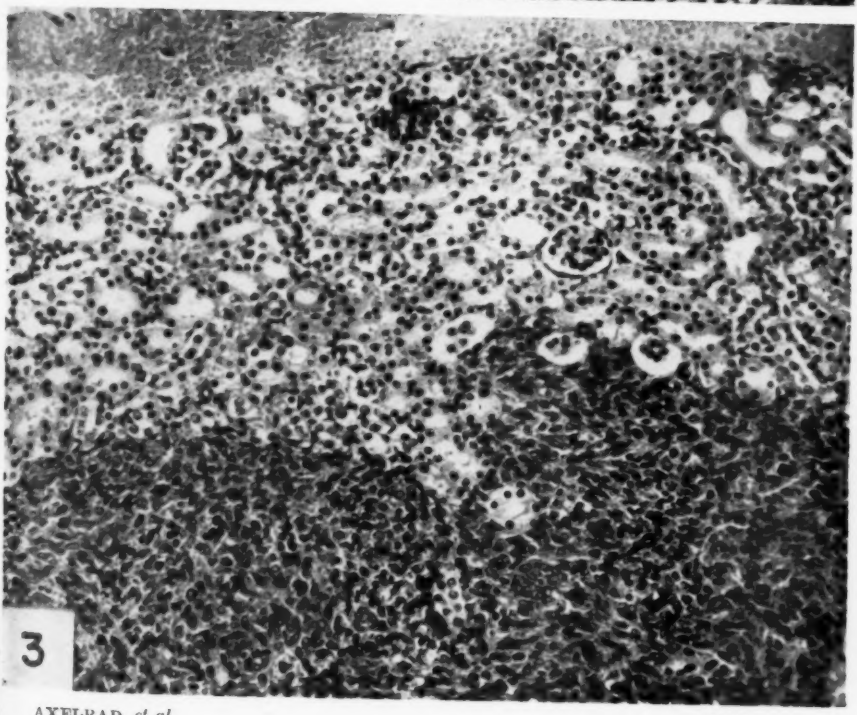

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PLATE 99

FIGURE 4.—Hamster that received injection of virus on 1st day of life and was dead at 21 days of age. *Note* irregular shape of right kidney due to presence of several nodular tumor masses, one of which is indicated by *arrow*. × 2

FIGURE 5.—Photomicrograph of nodular kidney tumor showing oval nuclei of sarcoma cells arranged roughly parallel to one another. This appearance is characteristic of both early diffuse and late nodular tumors. × 950

FIGURE 6.—Photomicrograph of 1 minute nodule of sarcoma in otherwise normal-appearing kidney tissue. × 350

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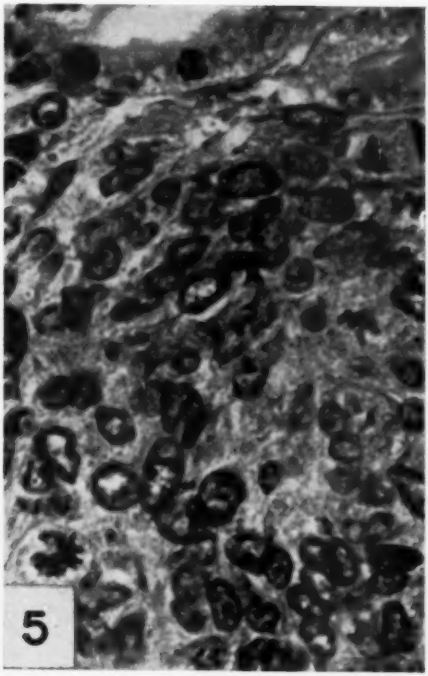

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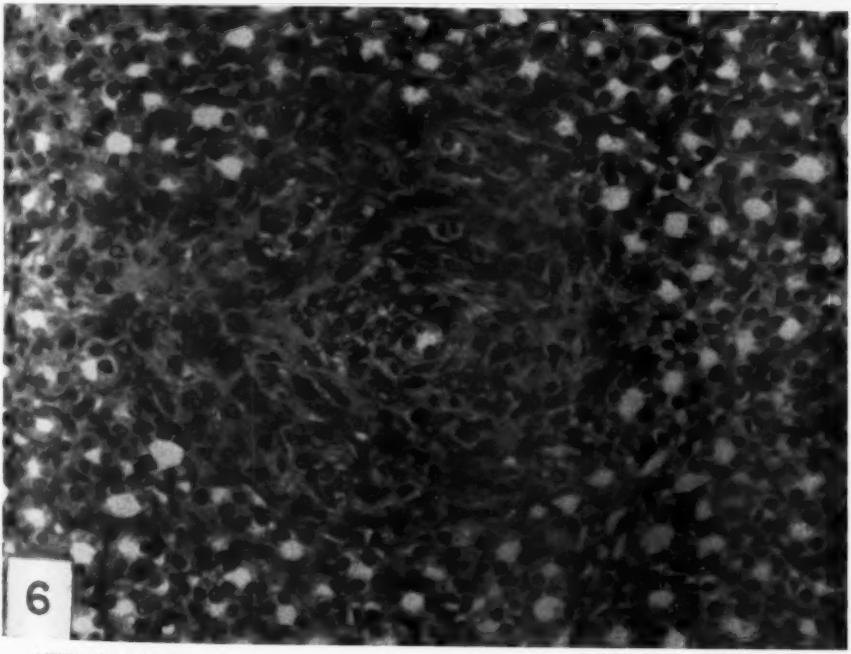

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PLATE 100

FIGURE 7.—Hamster that received injection of virus on 1st day of life and was dead at 3 weeks of age. *White arrows* indicate multiple discrete tumors of heart. *Black arrows* point to blood-filled blebs on surface of lung. × 2

FIGURE 8.—Photomicrograph of single tumor nodule in heart. × 210

FIGURE 9.—High-power photomicrograph of nodular heart tumor showing invasion of cardiac muscle by spindle-shaped cells. × 520

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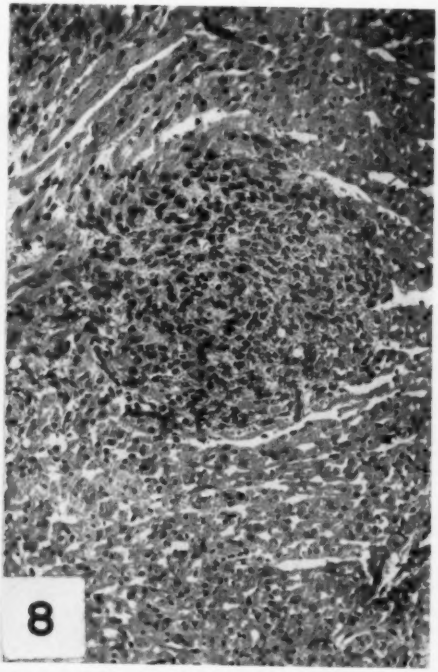

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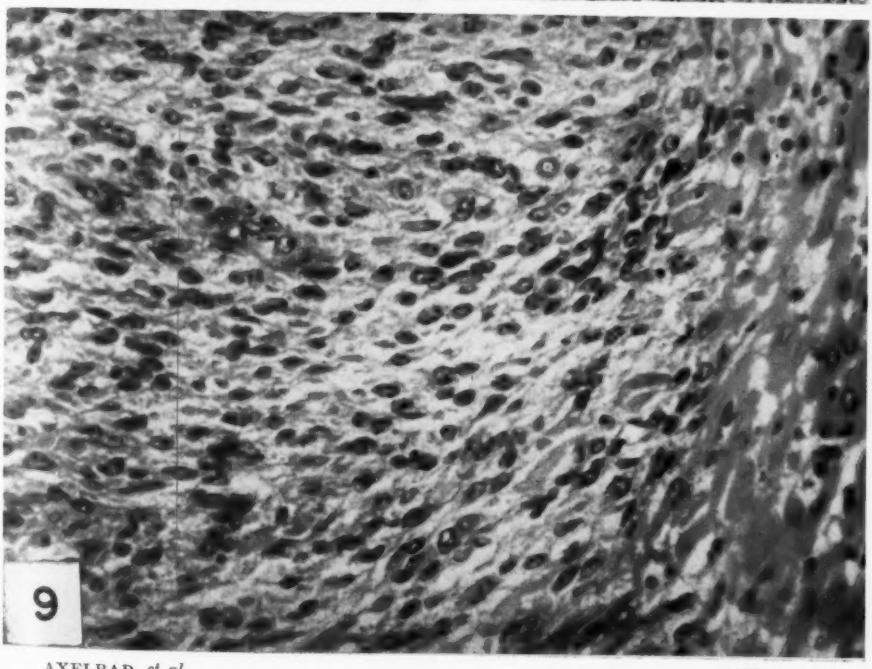

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PLATE 101

FIGURE 10.—Photomicrograph of blood-filled bleb at surface of liver. × 25

FIGURE 11.—Photomicrograph of blood-filled bleb at surface of lung. Inner wal ruptured and interstitial tissue infiltrated with red blood cells. × 100

FIGURE 12.—Photomicrograph of metastatic tumor nodule at periphery of lung of hamster bearing a massive diffuse kidney tumor. × 150

FIGURE 13.—Two discrete tumor nodules (*arrows*) attached to skin of control hamster that received normal monkey kidney culture supernatant fluid subcutaneously on 1st day of life and was killed at 5½ months. Actual size.

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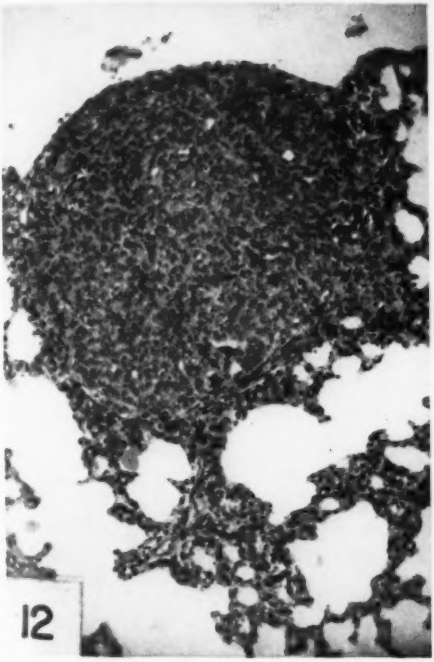
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The Histopathological Sequence in Viral Carcinogenesis in the Hamster Kidney [1, 2]

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SUMMARY

The histogenesis of a very rapidly developing kidney tumor of hamsters was investigated by studying sections of the kidneys of hamsters killed on successive days after they received virus. A distinctive lesion was seen at 4 days; this developed in and was confined to mesenchymal cells that were sufficiently undifferentiated or embryonal to serve as precursors of both the medullary stroma and the musculature of the walls of arteries and veins of the arcuate system that were still developing. The lesion was manifested by extensive and simultaneous proliferative and degenerative changes in these cells. By 6 days intranuclear inclusion bodies were apparent. Subsequently the degenerative response to the virus became decreasingly obvious, but since the proliferative response continued, the histological picture changed from one characteristic of a virus infection to one typical of neoplasia. There was no evidence to suggest that the neoplasia represented anything but a continuance of the proliferative response that began before the 4th day in a multitude of cells distributed widely throughout the kidney. The similarity of the two types of responses elicited in stromal cells to those observed in lysogenic systems of bacteria, where a virus may either destroy its host cell or change its genetic nature, is pointed out, and the significance of the type of cell that became infected, and the speed with which this occurred, to the problem of why the newborn animal should be peculiarly susceptible to tumor virus, is discussed.—J. Nat. Cancer Inst. 24: 1113–1129, 1960.

A VIRUS recovered from a mammary tumor of a C3H mouse, and transferred serially in cultures of mouse kidney cells, was found to induce kidney sarcomas in newborn hamsters so quickly that many animals died with tumors as early as 10 days after receiving the virus (*1*, *2*). This rapid and almost uniform induction of neoplasia provided a unique opportunity for studying the sequence of histopathological changes in an organ of a mammal, from the day it is exposed to a tumor virus until frank neoplasia is manifest. Accordingly hamsters were given the virus at birth and killed on successive days. Our histological observations on this material and our interpretations of these observations form the basis for this report. In a subsequent report (*3*), electron-microscope studies

[1] Received for publication September 28, 1959.

[2] This investigation has been supported by a grant from the National Cancer Institute of Canada.

of some of the virus lesions described in this report, as well as other details of the effects and properties of the virus that are relevant to establishing its relationship to the polyoma virus, will be presented.

MATERIALS AND METHODS

The origin and preparation of the virus used in this experiment are described in preceding reports (*1*, *2*). Two series of experimental animals were used, the first series consisting of 22 hamsters from 3 litters and the second of 18 hamsters from 2 litters. Each animal was injected subcutaneously with 0.2 ml. of the virus preparation within 24 hours after birth. Two to 5 hamsters were killed on the 1st, 2d, 3d, 4th, 5th, 6th, 7th, 8th, 9th, 10th, 11th, 14th, and 17th days after the injection. Uninjected animals of the same age were used as controls. All animals were autopsied immediately after death. Blocks from various organs were fixed in Bouin's fluid and sectioned in paraffin. Routine sections were stained with hematoxylin and eosin. Various other stains were used for special purposes.

OBSERVATIONS

Since the developing stroma of the renal medulla is the tissue primarily affected by the virus, its normal distribution and appearance in hamsters during the first few days of life will be described. The developing stroma is distributed so as to surround the tubules of the medulla at this time (fig. 1) but can be seen to best advantage in little patches that are scattered along the medullary side of the arcuate line (fig. 1). The patches are composed of mesenchyme which has two special features: (1) Many of the nuclei are oriented with their long axes parallel to the corticomedullary border (fig. 1). (2) A developing artery and vein of the arcuate system can be seen generally on the cortical side of each patch (fig. 1). The mesenchyme of the patches is continuous with the delicate sheaths of mesenchyme that invest the tubules of the medulla (fig. 1). The nuclei of the mesenchymal cells are ovoid, pale-staining, and contain one or more small nucleoli (figs. 3 and 4). The cytoplasmic outlines of the cells cannot be seen and most of the space between adjacent nuclei seems to be filled with ground substance (figs. 3 and 4).

The changes that were seen in this mesenchyme in the majority of animals at various days after they received an injection will be described first. In a smaller number of animals a somewhat different sequence of changes was observed and this will be described separately.

The first pathological change that occurred was observed in the kidneys of 1 of the 3 animals killed 3 days after receiving virus. The patches of mesenchyme were more prominent in this animal than in the control. In some parts of the patches, the cells were closer together and formed a denser type of stroma than in the normal animal.

In all the animals killed at 4 days, the following changes were observed in the stroma: (1) The amount of stroma had increased, which was evidenced not only by larger patches (fig. 2) but also by well-defined tongues of stroma extending from the patches into the medulla where they often formed septums between groups of tubules (fig. 2). (2) The stromal cells were packed more closely together (fig. 2) and many of their nuclei were damaged, being paler than usual except in the region of the nuclear membrane where their chromatin tended to be concentrated (figs. 5 and 6). In extreme instances the nuclei appeared as dark irregular rings enclosing nearly empty spaces (figs. 5 and 6). Aggregations of dark-staining debris were commonly observed between nuclei in areas where many nuclei had marginated chromatin (figs. 5 and 6).

There were many examples, at 4 days, of stromal cells abutting directly on the endothelium of vascular channels. Figure 7 represents a higher-power view of the developing vein seen in the upper left portion of figure 2, and shows that the stromal cells extend to the endothelium of the developing vein. It seems probable that under normal conditions these cells would have contributed to the smooth muscle of the vein wall. Figure 8 is a photomicrograph taken from an area in the medulla; it shows stromal cells abutting directly on endothelial tubes, with their long axes disposed at right angles to the tubes as if they were about to form the tunica media of circularly disposed smooth muscle cells of an artery.

Sections from kidneys of most animals 5 and 6 days after they received virus showed a further increase in the stroma, so that at this time large patches were numerous at and about the corticomedullary junction (fig. 9). Many of the patches were now elongated (fig. 9); some now extended into the cortex at sites where interlobular arteries are commonly seen. The majority of the cells of the stroma revealed nuclear damage. This was manifested by the chromatin being marginated leaving the more central part of the nucleus pale or empty. In scattered areas intranuclear inclusions were observed (figs. 10 and 11). The inclusion bodies were generally about half the diameter of the nucleus in which they were contained. Most were round but some were ovoid (figs. 10 and 11). Their substance appeared to be homogeneous. They were neither clearly basophilic nor acidophilic in routine sections stained with hematoxylin and eosin. In sections stained lightly with hematoxylin and fairly heavily with eosin or with phloxin they were pink, but in sections stained heavily with hematoxylin and lightly with eosin they were blue.

Although the majority of the nuclei of the stromal cells in these animals evidenced some damage, many had a healthy appearance and mitotic figures were commonly seen (fig. 10). Accordingly the diffuse lesion manifested in the stroma at 5 or 6 days was simultaneously degenerative and proliferative (fig. 10).

In the kidneys of most animals examined 8 days after injection there was a further growth of the stroma; by this time it occupied a much greater portion of the medulla than previously. Tongues of stroma could also be seen extending into the cortex between medullary rays. Although

many degenerating nuclei could still be seen, they were relatively less numerous than in the 6-day animals. A few scattered leukocytes were observed in the stromal tissue at this time.

After the 8th day there was considerable variation in the extent to which the lesions had progressed in different animals. In those kidneys that manifested the most advanced lesions, the altered stromal cells had grown progressively so as to form a large tumor mass (fig. 13) extending into and throughout most of the midzone of the medulla. The tumor, moreover, had extended into the cortex in many sites and the general picture was that of a diffuse mass of proliferating cells, with the cortical parenchyma spread thinly over it, infiltrating between such parenchymal units as persisted in the medulla. Mitotic figures were abundant (fig. 12). Some degenerating nuclei could still be seen but they were not nearly so numerous as in the earlier stages (fig. 12). One of the animals 11 days after receiving virus revealed a metastatic tumor in the lung, and other animals had primary tumors in their hearts, similar to those previously described (*2*).

While in the great majority of animals receiving an injection of virus there was a diffuse and generalized involvement of the developing stroma of both kidneys, obvious from 4 days onward, in a minority of animals only scattered small localized lesions were seen in the kidneys. Histologically these lesions in animals receiving virus 10 to 14 days previously were similar to those of animals receiving virus 4 to 6 days previously and in which widespread diffuse lesions developed in the kidney. These small localized and relatively slowly developing lesions would seem to be the forerunners of the nodular kidney tumors that have been observed to develop in some animals later than the diffuse tumors that develop in others (*2*).

In all instances if a widespread diffuse lesion was seen in one kidney, a comparable one was seen in the other; likewise if only small localized lesions were seen in one kidney, only this type of lesion was seen in the other.

DISCUSSION

It is pertinent to emphasize first, that the tumor whose development is described in this paper fulfills all the criteria of malignancy; it is locally invasive, gives rise to metastases, is transplantable, and because of its progressive growth kills the host in which it originates or into which it is transplanted (*2*); and second, the histopathology of the fully developed tumor gives no more suggestion of its being a virus-induced tumor than that given by the histopathological features of any malignant tumor, including those of man. However, when histopathological studies were made on kidneys 4 to 6 days after the animals were injected, the viral nature of the lesion was clearly apparent. Material obtained at this time showed that the lesion developed in and was confined to a particular type of cell and that the cells of this type responded to the virus by both proliferation and degeneration, and in many instances by the develop-

ment of intranuclear inclusions. The involvement of a special cell type, the character of the responses manifested by the cells of this type, and the development of intranuclear inclusions are all phenomena that have been variously observed in many common virus diseases.

The change in the histopathological picture from one indicative of virus infection to one typical of neoplasia took place over a period of a few days. The change occurred because, while the proliferation of the cells continued, degeneration almost ceased and cells that had already degenerated, disappeared. Accordingly our findings indicate that the typical neoplasia which developed was not due to the institution of a second and new process, but was a continuance of a process present from the beginning, a process which began in a multitude of cells scattered throughout the kidney and which we interpreted as a proliferative response of the cells to infection with virus.

Although proliferative or degenerative changes in cells infected by a virus have long been recognized as characteristic responses to virus, two features of the lesion that we observed make it unusual: (1) proliferative and degenerative responses appeared simultaneously and side by side in different members of the same cell family; and (2) with the passage of time the proliferative response emerged as the dominant one. A lesion with these two features would seem to provide another example of a virus-cell interaction to the spectrum already known in mammals.

How could the virus have evoked two types of responses in different members of the same family of cells? There is, of course, a biological precedent for this type of phenomenon. From the study of lysogenic systems of bacteria, it is known that a particular virus can produce two different types of effects on bacterial cells of the same kind. It can, under one set of conditions, proliferate within and destroy the cells, or under others, it can enter into the genetic apparatus of the cells to subsequently modify their hereditable nature. It may be, therefore, that the two types of responses observed in the stromal cell of the kidney were due to the virus either proliferating within the cells and destroying them, or entering into some kind of combination with or otherwise modifying their genetic material, so that thereafter the cells were neoplastic. Why certain cells should respond in one way, and others in another, is a further problem. Studies on lysogenic systems have shown that the physiological state of the bacterial cell can be the determining factor in whether it responds one way or the other, and it is possible that the different responses observed might be similarly explained as being due, for example, to cells becoming infected at different stages of their mitotic cycle or at imperceptibly different stages of differentiation. We have no information as yet on these matters; however, we do have reason to believe that the degree to which the stromal cells have differentiated is a factor in determining whether or not they are susceptible to the virus and that this in turn may be relevant to the peculiar susceptibility of newborn animals to tumor virus, as will become apparent from the next portion of the discussion.

The demonstration by Gross (*4*) that mouse leukemia could be transmitted by cell-free filtrates if newborn mice were used as recipients focused attention on the susceptibility of the newborn animal to tumor viruses and therefore was responsible for greatly broadening this field of research. It is of interest to ask why the newborn animal should be so susceptible. Why, for example, should the same dose of virus that produces kidney tumors when injected into newborn hamsters not do so when we give it to 3-week-old hamsters?

Before we made our histological studies on the early stages of the development of the kidney lesion we, like many others, assumed that the peculiar susceptibility of the newborn was probably related to the newborn animal being relatively unable to react to antigens by the production of antibodies. However, since we found extensive lesions in the kidney 4 days after the animals were given virus, and in one instance 3 days after, and since it would take some time for these lesions to develop, we can assume that the virus must gain entrance to the stromal cells very soon after it is given. This would give an animal so little time to make antibodies that it seems very probable that there must be some factor other than its inability to make antibodies that is responsible for the newborn being peculiarly susceptible to tumor virus. One suggested from our work—and there may be others—is that embryonal cells may be peculiarly susceptible to tumor virus and that their persistence in relatively large numbers in certain organs from fetal into early postnatal life may be concerned with the susceptibility of the newborn to tumor virus and with the organ in which tumors develop. As shown in figures 1, 2, 7, and 8, the distribution of the cells that become infected with virus suggests that they are the precursors not only of the ordinary stroma that invests the medullary tubules but also of the musculature of such blood vessels of the arcuate system as are developing at this time. The ability of any type of cell to form two different tissues, muscular and connective, is indicative of its possessing a great potentiality and, in general, being sufficiently undifferentiated to be considered as embryonal in nature.

If embryonal cells are peculiarly susceptible to tumor virus, it could be expected that in newborn animals given virus, tumors would most commonly develop in organs relatively undeveloped at birth and whose development continues into postnatal life, because it could be expected that in these organs very undifferentiated cells would have to persist in numbers until all parts of the organ were formed. The kidney and heart, the organs in which the virus commonly induces tumors in hamsters, and the salivary and mammary glands, the organs in which the virus commonly induces tumors in mice, are all characterized by not having completed their development at birth.[3]

[3] Although embryologists and histologists have long appreciated that some organs of the body have not completed their development at birth, and only do so in postnatal life, and that there are some variations in this matter due to species differences, information on this matter as a special topic has never been brought together and organized. Accordingly only isolated references can be given. For the postnatal development of salivary glands see Jacoby and Leeson (*5*). An account of the postnatal development of the mammary glands is given by Ham (*6*). The postnatal development of the kidney is described by Leeson (*7, 8*), and that of the impulse conducting system of the heart by Duckworth (*9*). An account of Duckworth's study is also given by Ham (*10*).

That embryonal cells are peculiarly disposed to develop into tumors is not a new thought. It will be recalled, for example, that Cohnheim's theory (*11*) was based on the concept that such cells served as the origin of tumors. Cohnheim, of course, believed that such cells were disposed to become neoplastic if they became displaced during embryonic development and hence were prevented from developing normally into differentiated parts of the organism. Our work likewise would seem to emphasize the importance of undifferentiated cells in tumorigenesis, but for another reason, because they are peculiarly prone to infection with tumor virus which acts on them both to interfere with their differentiation and to incite their neoplastic growth.

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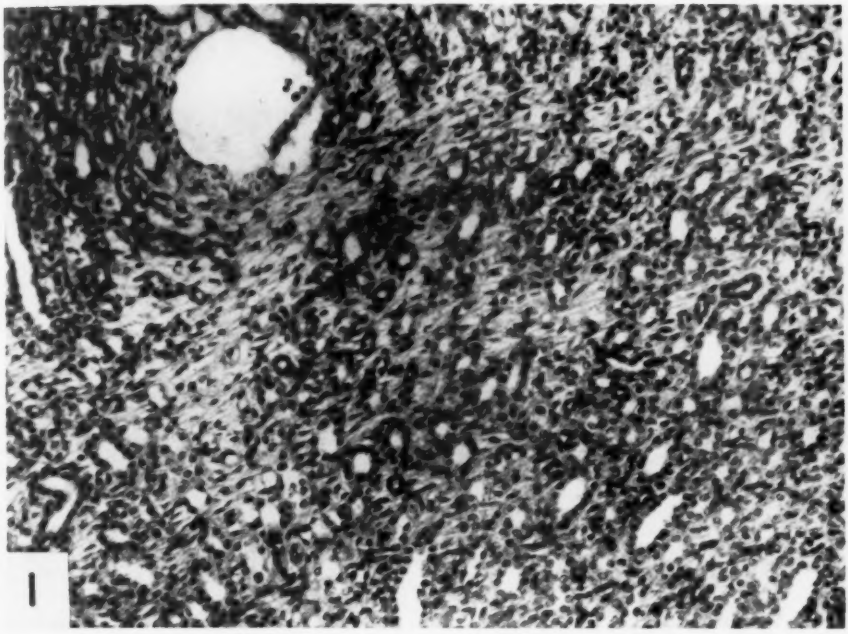
PLATE 102

FIGURE 1.—Section of kidney of normal hamster 4 days old. A developing vein may be seen in *upper left* of photomicrograph; vein is at corticomedullary junction. Patch of mesenchyme extends down from vein into medulla. Cells from patch extend between medullary tubules. × 170

FIGURE 2.—Section of kidney of hamster that was killed 4 days after receiving virus. Area illustrated is comparable to that in figure 1. *Note* that patch of mesenchyme extending down from vein is much more extensive than in normal hamster and that sizable tongues of tissue extend from it toward the right between groups of medullary tubules. × 200



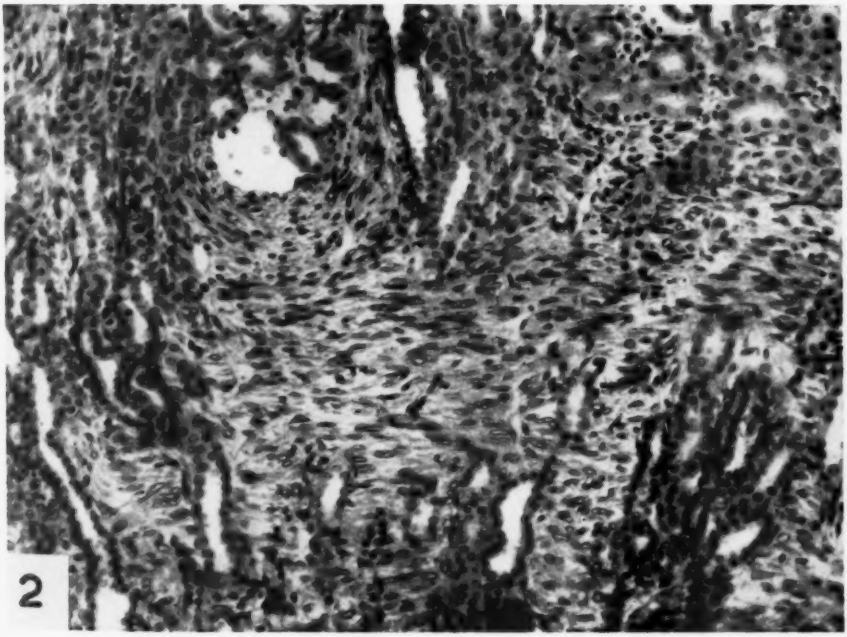


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PLATE 103

FIGURE 3.—Section of kidney showing details of mesenchyme in patch in 4-day-old normal hamster. × 400

FIGURE 4.—Section of kidney showing details of mesenchyme in patch in 4-day-old normal hamster. × 900

FIGURE 5.—Section of kidney showing details of mesenchyme in patch in hamster 4 days after receiving virus. Chromatin marginated to different extents in nuclei and debris between cells. × 900

FIGURE 6.—Section of kidney showing details of mesenchyme in patch in hamster 4 days after receiving virus. Nucleus at *lower right* almost empty and much debris above it. × 900 (Figures 4 and 6 taken at exactly same setting of camera.)

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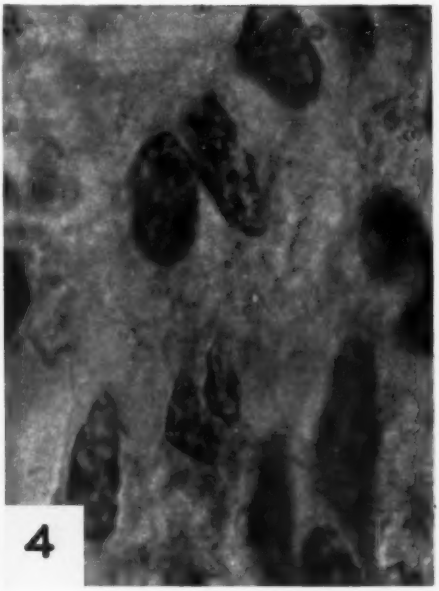
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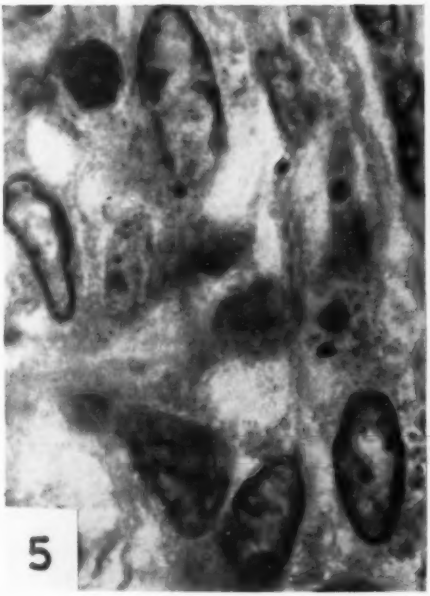
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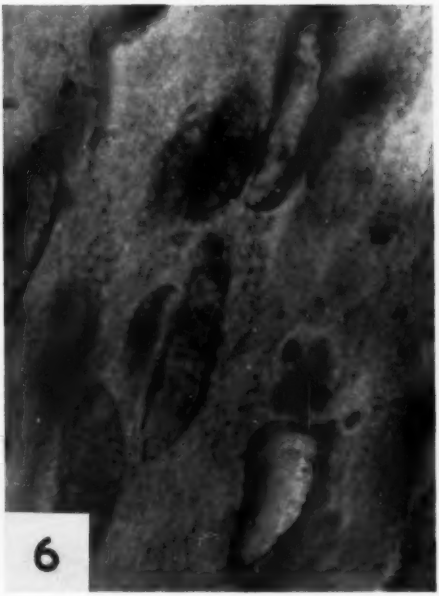
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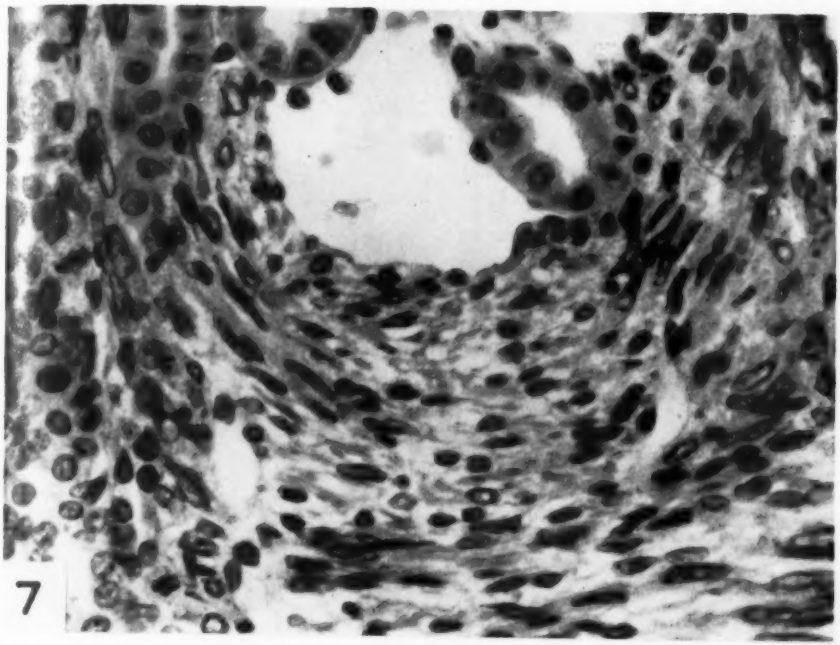
PLATE 104

FIGURE 7.—Higher-power view of developing vein and tissue beneath it, illustrated in figure 2. *Note* that mesenchyme of patch extends to endothelium of developing vein. × 400

FIGURE 8.—Area in patch farther down in medulla. *Note* that mesenchymal cells are disposed at right angles to long axes of endothelial tubes on which they directly abut. × 400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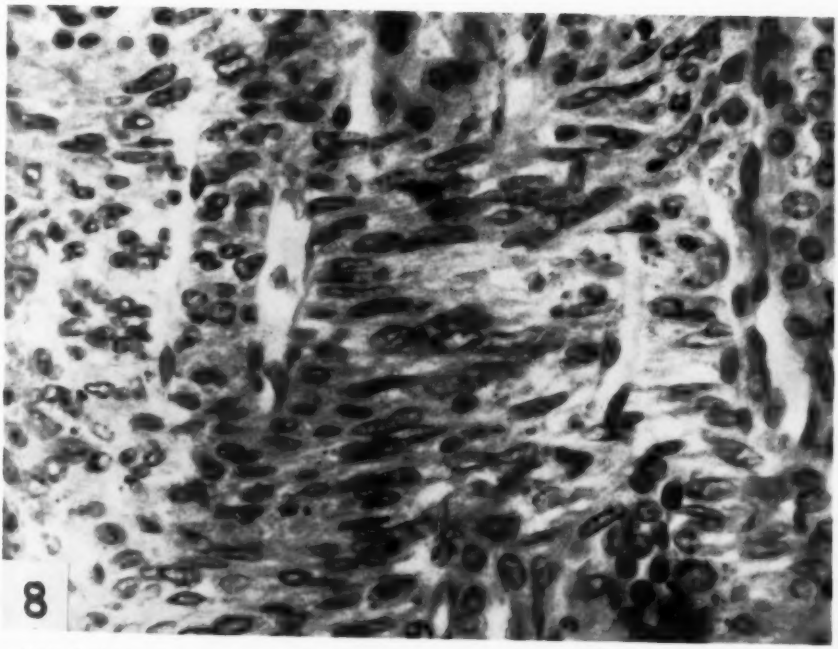
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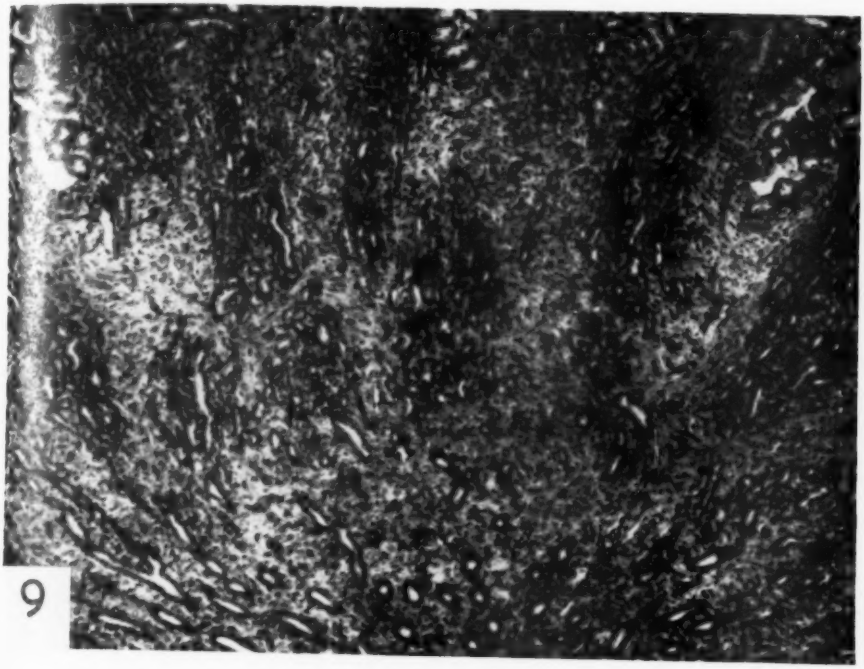
PLATE 105

FIGURE 9.—Area of medulla, close to cortex which may be seen above, in kidney of hamster given virus 6 days previously. Patches of mesenchyme greatly enlarged and fused. Stroma content of medulla as a whole increased. × 70

FIGURE 10.—Area from patch in hamster given virus 6 days previously. Intranuclear inclusion in *upper* part of picture and a mitotic figure near *lower left*. × 900

FIGURE 11.—Area in patch in hamster given virus 6 days previously. Intranuclear inclusion may be seen in *center*. Other nuclei show examples of marginated chromatin. × 900



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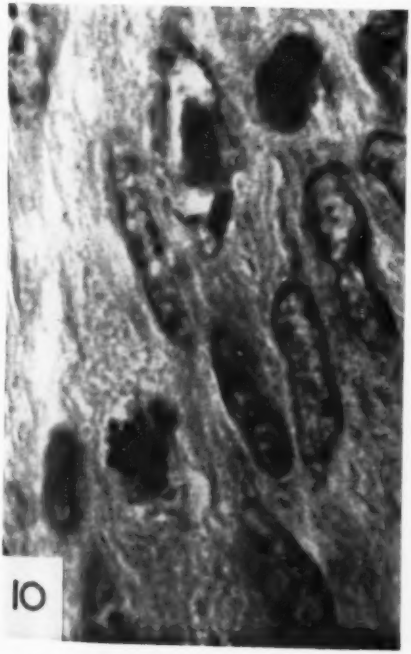

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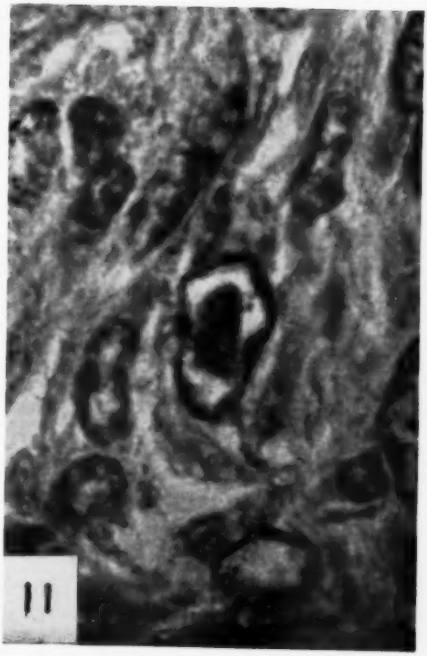

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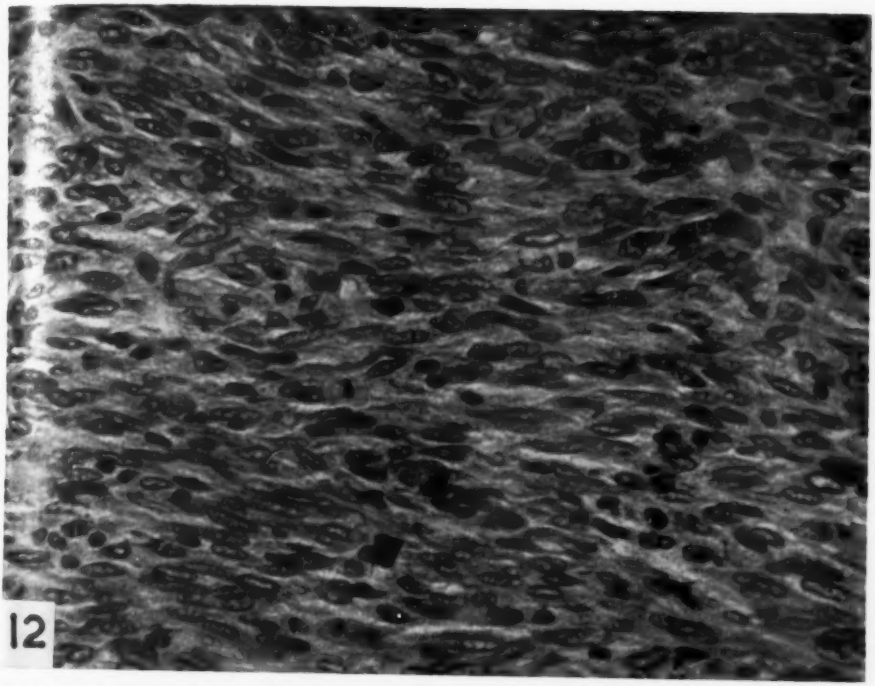
PLATE 106

FIGURE 12.—Area in an expanded patch in medulla in hamster given virus 9 days previously. Proliferative response now dominant and many mitotic figures can be seen. × 280

FIGURE 13.—Area at junction between cortex and medulla in hamster 11 days after receiving virus. Large tumor mass extends from medulla below into cortex above. × 75



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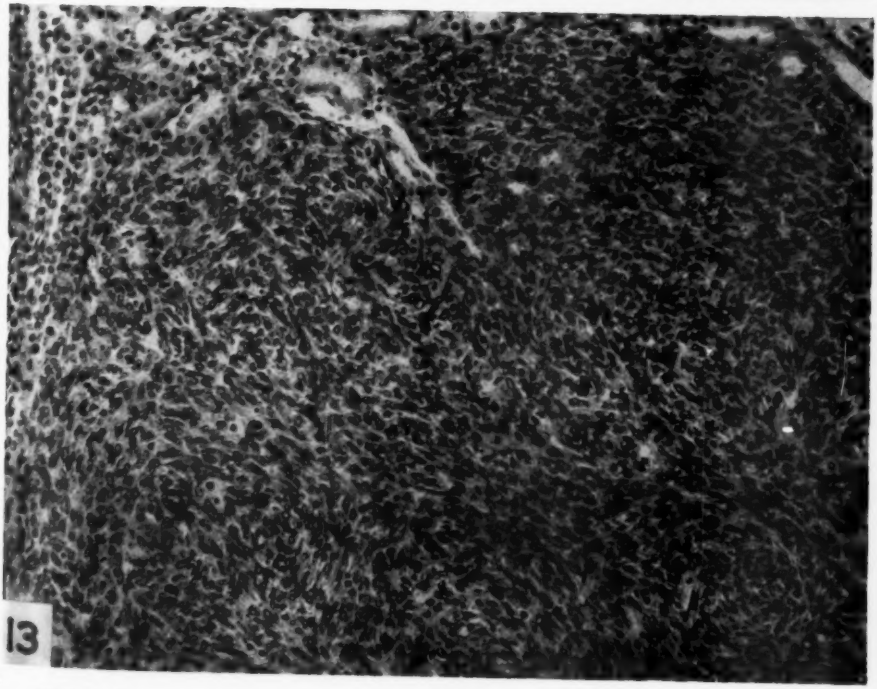

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Studies *In Vitro*, *In Vivo*, and by Electron Microscope of a Virus Recovered From a C3H Mouse Mammary Tumor: Relationship to Polyoma Virus [1, 2, 3]

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SUMMARY

Previous studies indicated that a virus we recovered in tissue culture from a C3H mouse mammary tumor had some effects in mice and hamsters that were similar to and others dissimilar from those of the polyoma virus. To clarify the relationship between these two viruses, further studies *in vitro*, *in vivo*, and with the electron microscope were made and are reported here. An important finding from the studies *in vitro* was that the two viruses were indistinguishable by hemagglutination inhibition tests. The studies *in vivo* related chiefly to the effects of the virus in rats; this had not previously been determined. Our virus was found to be much more pathogenic for this species than the polyoma virus was. Finally, electron-microscope studies were made on hamster kidneys 5 to 7 days after infection; previous light-microscope studies had shown that the necrotizing effect of the virus was then at its peak. These studies revealed particles 28 mμ in diameter particularly in the nuclei and in phagocytosed material in the cytoplasm of medullary stromal cells. Similar particles were found in nuclei of cells in which the virus was being propagated, and a few were occasionally seen in the nuclei of cells of parotid tumors induced in mice by the virus. These particles probably represent the virus responsible for the tumors; their morphology is similar to that described by others for the polyoma virus. The significance of larger viruslike particles observed in some of the tumors induced in mice is discussed. The conclusion from all our studies is that the virus recovered from the C3H mammary carcinoma belongs to the polyoma family. However our virus preparations are less carcinogenic for mice and more carcinogenic for hamsters and rats than the polyoma virus recovered by Stewart, Eddy *et al.* These differences suggest that the virus recovered in our laboratory is a variant of the polyoma virus.—J. Nat. Cancer Inst. 24:1131–1151, 1960.

IN A preliminary report (*1*) we described some effects *in vitro* of a virus recovered from a mammary tumor of a C3H mouse and gave preliminary data on its tumor-inducing effects in Swiss mice and hamsters. In two subsequent papers (*2*, *3*) we presented details of its effects on hamsters.

[1] Received for publication November 23, 1959.

[2] This investigation has been supported by a grant from the National Cancer Institute of Canada.

[3] The term "polyoma virus" is used to describe a virus recovered from tissue cultures that had been inoculated with extracts containing the parotid tumor virus of Gross. The terms "polyoma virus" and "parotid tumor virus" are sometimes used interchangeably.

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Although we noted in the first report that the effects of the virus *in vitro* and *in vivo* had much in common with those reported for the polyoma virus, we have hesitated to draw more definite conclusions about the relationship of this virus to the polyoma virus until we had completed the work described in this paper. As will be described, the results of our experiments *in vitro* and our studies with the electron microscope indicate that a virus of the polyoma family was present in our extracts. However, the results of injecting our preparations into newborn mice, hamsters, and rats, some of which are being reported for the first time in this present paper, indicate that the pathogenicity of our preparations differs sufficiently from that reported for the polyoma virus, described by Stewart and Eddy *et al.* (*4–8*), to indicate that we probably have a variant of this virus.

MATERIALS AND METHODS

As previously described in some detail (*1*), an extract prepared from a mammary tumor in a C3H/Jax mouse was added to a culture of C3Hf/HeHa mouse kidney cells, producing a cytopathogenic effect. The virus has been passed serially in cultures through many transfer generations.

For the tests *in vivo* Swiss ICR mice, Syrian hamsters, and Wistar rats were used. The Swiss ICR mice were randomly bred in this laboratory. Injections of 0.05 ml. of virus preparation were given subcutaneously within 48 hours after birth. The mice were weaned at 3 weeks and fed *ad libitum* on Ralston Purina Mills Laboratory Mouse Diet.

Pregnant hamsters from High Oak Ranch, Richmond Hill, Ontario, were delivered to our laboratory 1 or 2 days before they littered. The young were injected subcutaneously with 0.2 ml. of virus preparation within 24 hours after birth.

Pregnant Wistar rats were obtained from Woodlyn Farms, Guelph, Ontario. These littered 2 days after arrival, and the newborns were injected subcutaneously within 24 hours after birth with 0.2 ml. of a virus preparation known to induce tumors very rapidly in hamsters.

As controls, mice and hamsters similar to the experimental animals were injected with fluid taken from normal cultures of C3Hf kidney cells that showed no evidence of degeneration after 2 weeks of cultivation.

Tissue for electron microscopy was fixed in 1 percent buffered osmium tetroxide, dehydrated in alcohol, and embedded in methacrylate. Thin sections were cut with a Porter-Blum microtome and examined in an RCA EMU 3b electron microscope. Some of the sections were stained with lead hydroxide, after the method described by Watson (*9*).

For hemagglutination tests, guinea-pig or rabbit red cells were washed in phosphate-buffered saline and mixed with dilutions of the virus. The tubes were kept at 4° C. for 2 to 24 hours before being read.

Antiserum to the virus was prepared by injecting tissue-culture lysate intravenously into New Zealand white rabbits 3 times a week for 3 weeks.

The rabbits were then bled by cardiac puncture. Serum from 5 rabbits was pooled and from this a semipurified γ-globulin was prepared by alcohol fractionation (*10*).

Hemagglutination inhibition tests were carried out with serial dilutions of γ-globulin in phosphate-buffered saline mixed with a standard amount of virus and incubated for 1 hour at 37° C. Guinea-pig red cells were then added and hemagglutination tests carried out as before.

EXPERIMENTS AND RESULTS

Properties of the Virus *In Vitro*

The cytopathogenicity of the virus was tested on cultures of cells derived from various sources (table 1). Cytopathogenic effects were observed regularly in every type of mouse cell tested, with the exception of Earle's strain L cells. Cytopathogenic effects were observed in mouse cells whether the animal from which they were obtained did or did not carry the milk agent. Normal cells from the monkey, rabbit, rat, and hamster were not visibly affected by the virus; hence the cytopathogenic effect of the virus appears to be specific for mouse cells. This species specificity in tissue culture contrasts with the wide spectrum of species in which the virus induces tumors. Further, although hamster cells are not destroyed by the virus *in vitro*, one response observed *in vivo* was destruction of hamster renal mesenchyaml cells (*3*).

TABLE 1.—Visible effects of virus on various cells cultivated *in vitro*

Cell cultures showing cytopathogenic effect		Cell cultures showing no cytopathogenic effect	
Mouse cells:	C3Hf/HeHa kidney	Mouse cells:	Earle's strain L
	C3H/StHa kidney	Hamster cells:	Adult kidney
	Swiss/ICR kidney		Embryo kidney
	Swiss/ICR embryo		Embryo heart
	C3H mammary tumor		Whole embryo
	DBA mammary tumor	Rat cells:	Kidney
		Rabbit cells:	Kidney
		Monkey cells:	Kidney

The virus is still cytopathogenic after being kept at 10° C. for 3 months, 37° C. for 3 hours, or 57° C. for 30 minutes. It agglutinates red cells from rabbits and guinea pigs. When a cytopathogenic effect is complete, the virus-containing fluid from the culture usually gives a hemagglutination titer of around 1:640, but occasionally much higher titers are obtained.

Hemagglutination by our virus and by the polyoma virus [4] is inhibited by γ-globulin prepared from serum of rabbits immunized with our virus. Also γ-globulin prepared from the serum of rabbits immunized with the polyoma virus inhibits hemagglutination by our virus as well as by the

[4] Sent to us by Dr. Bernice Eddy.

polyoma virus. Antihemagglutination titers in all these tests lay between 1:10,000 and 1:300,000. Dr. W. P. Rowe (*11*) has tested our virus in a 1:400 dilution of serum from mice immunized against the polyoma virus and found inhibition of hemagglutination.

Properties of the Virus *In Vivo*

Mice.—Preliminary information on the induction of tumors in Swiss mice by the virus has previously been presented (*1*). Since that time, more data have become available and the full results on the types of tumors observed and on their incidence are shown in table 2. In table 3 the latent interval for the appearance of tumors in the mice is shown.

Fifty-nine percent of the mice in the group that received virus developed tumors—parotid carcinomas, generalized lymphomas, or mammary carcinomas; the last occurred in virgin females. Only 1 mouse had more than 1 type of tumor and this animal had all 3 types. A few tumors occurred in the control animals; these were parotid tumors and lymphomas, with 1 mouse having both types. The final tumor incidence in the controls, including some late lymphomas discovered when the experiment was terminated after 13 months, was 18 percent.

TABLE 2.—Incidence of tumors in Swiss mice injected at birth with fluid from virus-infected and normal cultures of C3Hf kidney cells

Injected with:	Number surviving 4 months	Number with tumor	Tumor type		
			Parotid	Lymphoma	Mammary
Fluid from virus-infected cultures	44	26	12	11	5
Fluid from normal cultures	49	9	4	6	0

TABLE 3.—Latent interval for appearance of tumors in Swiss mice injected at birth with fluid from virus-infected and normal C3Hf kidney cultures

Injected with:	Tumors	Latent interval (*months*)		
		First tumor	50 percent incidence	Last tumor
Fluid from virus-infected cultures	Parotid	5	6	11
	Lymphoma	7	11	13
	Mammary	4	6	11
Fluid from normal cultures	Parotid	6	6	7
	Lymphoma	7	13	13

Hamsters.—The results of injecting newborn Syrian hamsters with our virus preparations have been described in detail (*2*, *3*). In brief, 10 to 16 days after injection many of the animals die with huge bilateral renal sarcomas, and within a month almost 100 percent die with sarcomas of the kidney, heart, or both.

Rats.—Thirty-one newborn rats, from 3 litters, were injected with virus subcutaneously, and thereafter inspected daily. Eight rats were lost early in the experiment and no data are available on these. The remaining animals died or were killed. Animals found dead were autopsied. Animals with obvious signs of neurological lesions or palpably enlarged kidneys were killed. The experiment was terminated on the 40th day when the remaining animals were killed. Twenty-one of 23 rats that died or were killed had gross renal tumors, and 1 had a microscopic renal tumor. The earliest death from a tumor occurred 25 days after injection of the virus, and massive kidney tumors were found in most of the others 30 to 40 days after they received an injection (figs. 1 and 2).

The kidney tumors were sarcomas and had a microscopic appearance similar to that observed in hamsters infected with the virus (fig. 3). Four rats had intracranial hematomas. These lesions were found in animals showing neurological signs of disease. The brains were not examined in the other animals. Small subcutaneous hematomas were found occasionally, but no other hemorrhagic lesions were seen.

Electron Microscopy

Electron-microscope studies were made on the following material: (1) the mammary tumor from which the original extract was prepared; (2) tumors induced in Swiss mice and hamsters by the virus preparation; (3) cultures of mouse kidney and mouse embryo cells on which the virus was being propagated; and (4) kidneys of 5- to 7-day-old hamsters that were injected shortly after birth with the virus preparation.

The tumor which was the source of the original extract had an appearance in the electron microscope similar to that of other milk agent-induced mammary tumors; the features of these have been described by a number of authors (*see 12*). The two distinct types of particles associated with these tumors, and referred to by Bernhard (*12*) as Types A and B, were present. The Type A particles occurred in unusually large numbers, forming dense juxtanuclear aggregates or inclusions. When the cells were arranged in alveoli the inclusions were seen in the cytoplasm on the side of the nucleus adjacent to the lumen, the site occupied in the normal cell by the Golgi apparatus (fig. 4). At higher magnifications (fig. 5) the individual particles in the inclusions presented a vesicular appearance, each consisting of a matrix of low density and a dense double-layered limiting membrane. The external diameter, which was uniform, was about 70 mμ. Type B particles were observed in much smaller numbers; most of them were extracellular and either attached or close to a free cell surface or scattered within alveolar lumens. These particles were larger than the Type A particles, varied more in size, and had a mean diameter of about 110 mμ. Most of them contained a dense, usually eccentrically placed, nucleoid (fig. 6). No viruslike particles were observed within the nuclei of the tumor cells.

Twelve of the tumors (5 parotid tumors, 4 mammary tumors, and 3 lymphomas) induced in Swiss mice were examined in the electron microscope. No intracellular inclusions similar to those observed in the original mammary tumor were seen. However particles about 110 mμ in diameter were observed in large numbers in 2 parotid tumors and in 1 mammary tumor, and in smaller numbers in 2 other parotid tumors and 1 other mammary tumor (fig. 7). The particles were extracellular and usually concentrated within lumens in the more differentiated parts of the tumors. Few or no particles of this type were observed in the more anaplastic tumors, or in the more anaplastic portions of the more differentiated tumors. These particles were of the type described by Bernhard in tissues of mice with spontaneous and virus-induced leukemia and referred to by him as Type C particles (*13*). It should be emphasized that they are different from the extracellular particles of the tumor of origin in that they are more uniform in size and possess larger, less dense, and more centrally placed nucleoids.

An examination of mouse kidney and mouse embryo cells from cultures in which the virus was being propagated revealed no particles that could be identified with those described in the tumor of origin or in the induced tumors. Subsequently, however, intranuclear particles of smaller dimensions were observed in some of these cells; these will be described.

As previously reported (*1*, *2*) the injection of the virus into newborn hamsters resulted in the rapid production of renal lesions. At first the lesions were characterized by degeneration of some cells and proliferation of others. Later the proliferative response became dominant. Electron-microscope studies were made on hamster kidney tissue obtained at daily intervals after virus injection. Attention was directed mainly toward the period 5 to 7 days after injection when observations with the light microscope showed that the necrotizing effects of the virus were at their peak (*3*). In kidneys of animals killed at this stage the developing stroma of the medulla near the cortex was much more extensive than in the normal kidney. There was a considerable amount of cell debris and intranuclear inclusions were observed in some cells. In the electron microscope many of the stromal cells showed evidence of pathological changes, such as margination and increased density of the chromatin and swelling of cytoplasmic components, but no detectable viruslike particles. In a few scattered areas, however, there were groups of stromal cells that showed similar pathological changes but, in addition, contained within their nuclei aggregates of particles of a uniform size. These particles showed no distinct limiting membrane, were usually of uniform density, and had a diameter of about 28 mμ[5] (figs. 9 and 10). It will be convenient to refer to them as Type D particles.

In some infected cells, arrays of closely packed particles, which in places showed evidence of crystalline arrangement (fig. 10), completely filled the nuclei; but in the nuclei of other cells the particles were less regularly arranged and less tightly packed, and dense material, probably

[5] In sections stained with lead hydroxide the particles appeared somewhat larger.

chromatin, was also present (fig. 9). Aggregates of viruslike particles were found even more abundantly as inclusions in the cytoplasm of phagocytic cells present in the same areas as cells that contained intranuclear virus (fig. 12). Some of these inclusions consisted entirely of the small particles already described, but in others larger particles were also seen. These had a dense core 28 to 30 mμ in diameter surrounded by a clear zone and a dense limiting membrane; their over-all diameter was about 60 mμ. The relationship of these particles to the Type D particles is being investigated.

The finding of particles in the nuclei of infected hamster kidney cells suggested that a further search should be made in the infected tissue-culture cells from which the virus preparation was obtained, to determine if similar particles could be seen in their nuclei. When this was done, intranuclear particles similar in size and appearance to those observed in the nuclei of the kidney cells of the infected hamsters were observed in cultures of mouse kidney cells that showed cytopathogenic effects 4 to 6 days after inoculation with virus-containing fluid (fig. 11). A further examination of some of the tumors induced in Swiss mice also revealed, in a few cells, small intranuclear aggregates of similar particles (fig. 8).

The electron-microscope findings may be summarized as follows: In the original mammary tumor the two types of particles repeatedly observed in milk-agent-induced tumors were present—the intracellular vesicular or doughnut type (Type A) with a clear center, double limiting membrane, and over-all diameter of 70 mμ, and the extracellular type (Type B) with a small, dense eccentrically placed nucleoid and an average diameter of 110 mμ.

In the tumors induced in Swiss mice two types of particles were found, both distinctly different from those observed in the original tumor. The first type, Type C, was about 110 mμ in diameter and had a relatively large (60 to 70 mμ) centrally placed nucleoid of variable density. The second, Type D, was much smaller (28 mμ). It usually had uniform density and was seen in the nucleus.

In infected tissue-culture cells the only type of particle seen in large numbers was the small intranuclear Type D particle.

In the hamster kidney lesions the Type D particles were present in great numbers, but larger particles 60 mμ in diameter, possibly related to the Type D particles, and distinct from the other particles previously described, were also present and particularly obvious in phagocytosed material.

Thus the only type of particle that occurs regularly in cells known to be infected with the virus is the Type D particle.

DISCUSSION

Relationship to Polyoma Virus

In view of the origin of our virus, perhaps the first point to establish is that although we were able, with the electron microscope, to demon-

strate the types of particles generally accepted as representative of the milk agent in the tumor from which the original extract was made, we were unable to demonstrate particles of this type either in the cells in which our virus was propagated or in the lesions induced by the virus in animals. There was, however, abundant evidence from our studies indicating that a polyoma-type virus was present in our virus preparations and was responsible for the results obtained. That a polyoma-type virus might be recovered from a C3H mouse mammary tumor is understandable in view of the finding of Rowe *et al.* (*14*) that polyoma virus is endemic in many colonies, including the one from which we obtained the mouse serving as the source of our virus.

Our virus, like the polyoma virus, is cytopathogenic for mouse cells in tissue culture, causes agglutination of erythrocytes, is able to withstand relatively high temperatures, and induces tumors in Swiss mice, hamsters, and rats. Moreover, by hemagglutination inhibition tests the two viruses are serologically indistinguishable.

Our recent electron-microscope findings are also consistent with the conclusion that the virus is of the polyoma type. Until recently, information about the size and morphology of the polyoma virus was based on filtration and centrifugation data, and was scanty and even contradictory. More accurate information was first provided by Kahler *et al.* (*15*), who obtained characteristic particles from polyoma virus-infected tissue-culture fluids after purification by density gradient centrifugation. In shadowed preparations the particles measured 44 mμ in diameter and the number of particles was directly proportional to the hemagglutination titer of the virus. Very recently Bernhard *et al.* (*16*), Banfield *et al.* (*17*), and Dmochowski *et al.* (*18*), have described particles about 27 mμ in diameter in sections of mouse cells that were infected *in vitro* with the polyoma virus. Dmochowski (*18*) has also described similar particles in tumors induced in mice by the polyoma virus. Though morphological observations alone cannot provide conclusive proof, there seems little doubt that the particles described by these authors represent the polyoma virus. The difference in size between the shadowed and sectioned particles, though large, may well be due to the different techniques employed.

The particles that we described as Type D appear to be indistinguishable from those observed by Bernhard *et al.*, Banfield *et al.*, and Dmochowski *et al.* This lends weight to the general conclusion that our virus is a member of the polyoma family.

There remain, however, important differences between our results and those reported for the polyoma virus isolated by Stewart, Eddy *et al.* (*6–8*). Our virus preparations produced fewer tumors and fewer types of tumors in Swiss mice than the polyoma virus did. That these differences were not due to differences in the animals but to differences between the viruses is suggested by the fact that the polyoma virus supplied to us by Dr. Eddy induced more tumors, with a wider spectrum of tumor types and a shorter latent interval, in the Swiss mice of our colony than our virus induced. In hamsters and rats, however, our virus was distinctly more

pathogenic than the polyoma virus. Our results in hamsters have been compared in detail with those of Eddy *et al.* in a preceding paper (*2*). Briefly, massive, diffuse bilateral renal sarcomas developed very rapidly in nearly 100 percent of our animals; Eddy *et al.* obtained nodular kidney tumors in fewer animals after a longer latent interval. The contrast between the effects of the two kinds of preparations is equally marked in rats. Ninety-two percent of our animals had gross tumors by 40 days after injection of virus, and the first animals died with tumor after 25 days, while Eddy *et al.* observed 18 tumors in 65 animals injected (36%), and the first deaths occurred 56 days after injection. Finally, no intracranial hemorrhagic disease similar to that which occurred in our rats was mentioned by Eddy *et al.* Compared with the polyoma virus, then, our preparations are more carcinogenic for hamsters and rats and less carcinogenic for mice.

The conclusion from all our studies is, therefore, that the virus recovered from a C3H mammary tumor and propagated in tissue culture on mouse cells is probably a variant of the polyoma virus.

Type C Particles and Lymphomas

The use and limitations of the electron microscope as an aid in identifying viruses have been discussed by Williams (*19*). As Williams points out, the presence of viruslike particles within or associated with the cells of a virus-induced lesion does not necessarily indicate that these particles are the causative agents. This is well illustrated by our observations. It appears from our more recent work that the Type C particles that were the most prominent ones in the tumors induced in mice and illustrated in our first report (*1*) are probably not the agents inducing the tumors. One possibility is that these particles are not virus but represent a reaction of the cells to some abnormal condition. In seems more probable, however, that these are virus particles; they resemble the particles often seen in tumors induced by the Gross leukemia virus. Other indications that leukemia virus was present or came to be present one way or another in these mice was the development of several lymphomas in our experimental series and an appreciable number also in the mice injected with control tissue-culture fluid. Gross (*20*) and, independently, Lieberman and Kaplan (*21*, *22*) have shown that a leukemogenic virus could be recovered from mice of low leukemic strains in which leukemia had been induced by radiation. Others have found an increased incidence of lymphomas in mice injected with control tissue-culture fluid (*23*, *24*). These findings suggest that leukemia virus in some form may be harbored in many kinds of mice and may be activated by the experimental procedures either in tissue culture or in the animals. Finally, it is possible that our polyoma virus induced the lymphomas; if so, this represents another respect in which our variant differs from the virus isolated by Stewart, Eddy *et al.* since they did not report the induction of lymphomas by their preparations.

Virus-Cell Relationships

In a previous paper (*3*) it was suggested that the virus could affect the stromal cells of the hamster kidney in two ways: it could proliferate within and destroy them; or it could enter into some kind of combination with the genetic material of the cells in such a way as to subsequently modify their behavior so that they become neoplastic. If the latter should occur it seems probable that the virus would lose its identity as such. The electron-microscope findings are in accord with this in that we have been able to demonstrate the virus with relative ease in infected tissue-culture cells and degenerating hamster mesenchymal cells in which the virus is multiplying and causing necrosis, but to date we have been unable to demonstrate it in the tumors induced by the virus in hamster kidney and only occasionally in the cells of the parotid tumors induced in mice by the virus.

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PLATE 107

FIGURE 1.—Rat injected with virus on 1st day of life and killed at 35 days of age. Kidneys are massively enlarged.

FIGURE 2.—Cut surfaces of kidneys of rat injected with virus at birth. Most of renal parenchyma replaced by tumor.

FIGURE 3.—Microscopic appearance of typical renal sarcoma induced in the rat by virus. × 900

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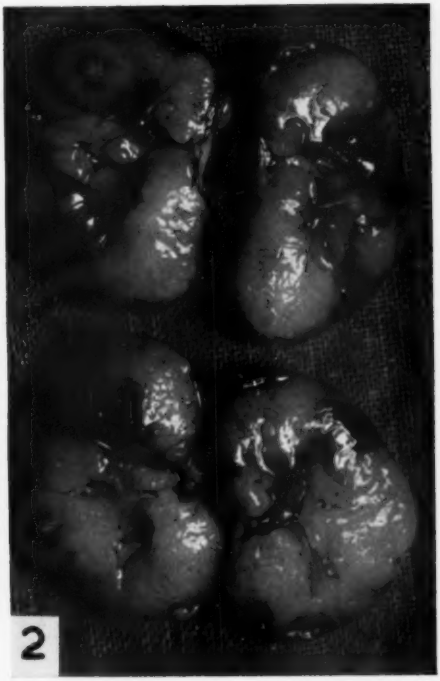
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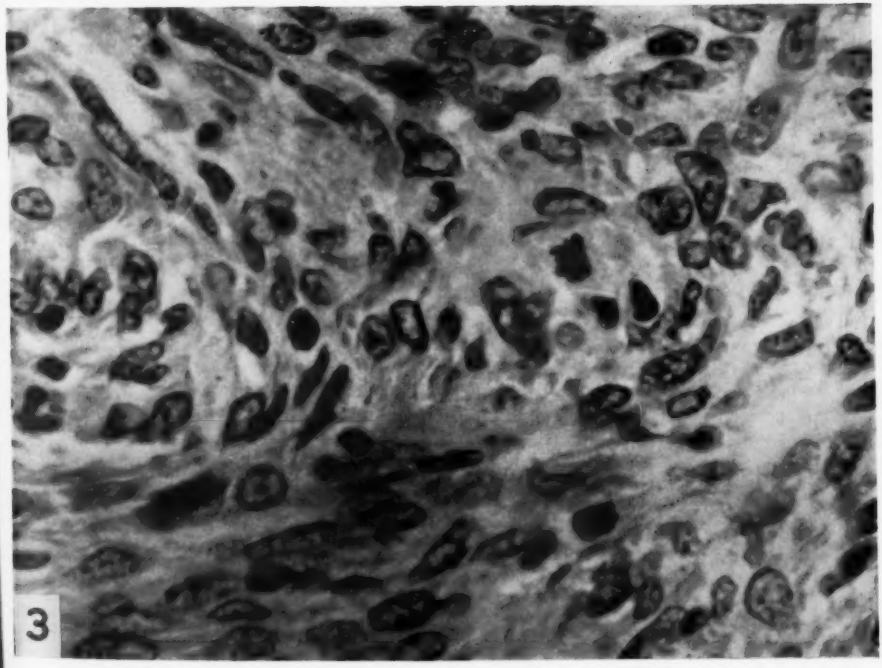
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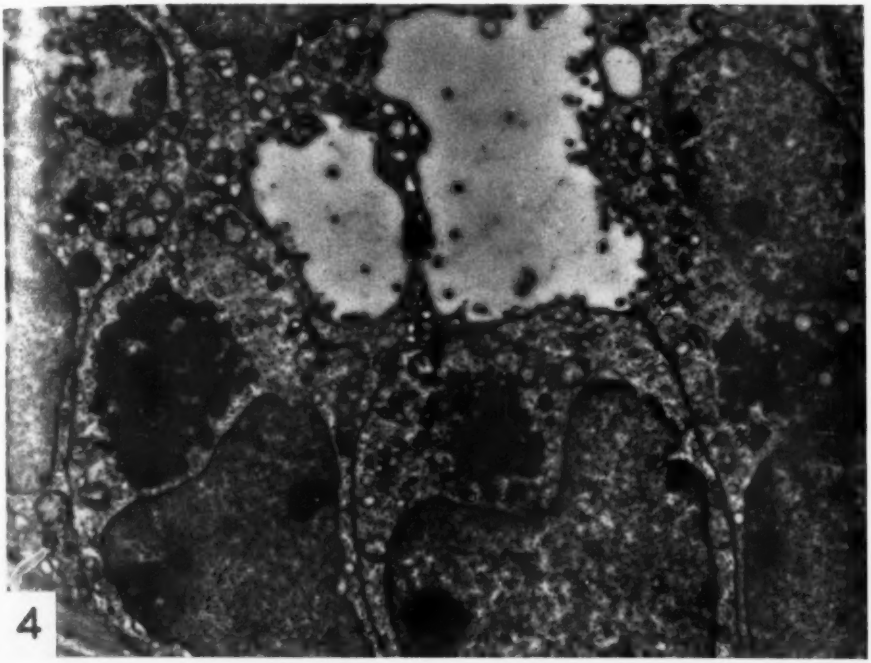
Figures 4 to 12 are electron micrographs. Of these, figures 5, 9, 10, 11, and 12 are from sections treated with lead hydroxide.

PLATE 108

FIGURE 4.—Section of C3H mammary tumor from which virus preparation originated. Part of an alveolus showing several cells with dense, juxtanuclear cytoplasmic inclusions, and scattered particles within lumen. × 11,500

FIGURE 5.—Detail of cytoplasmic inclusion showing structure of individual Type A particles. × 80,000

FIGURE 6.—Micrograph showing structure of Type B particles in C3H mammary tumor. × 75,000



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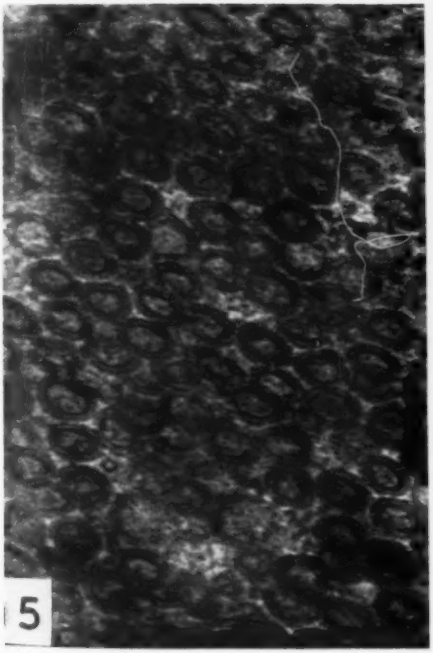

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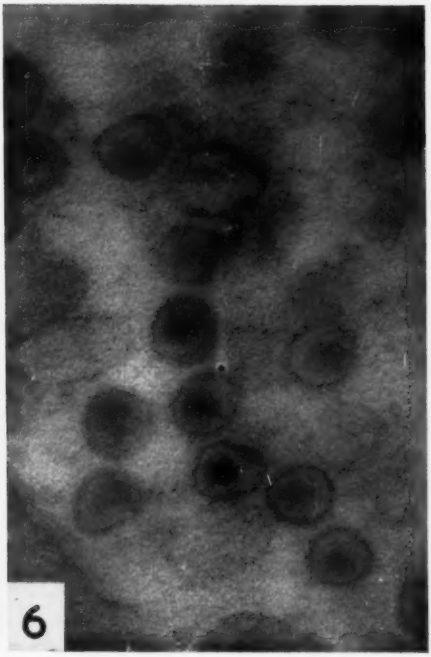

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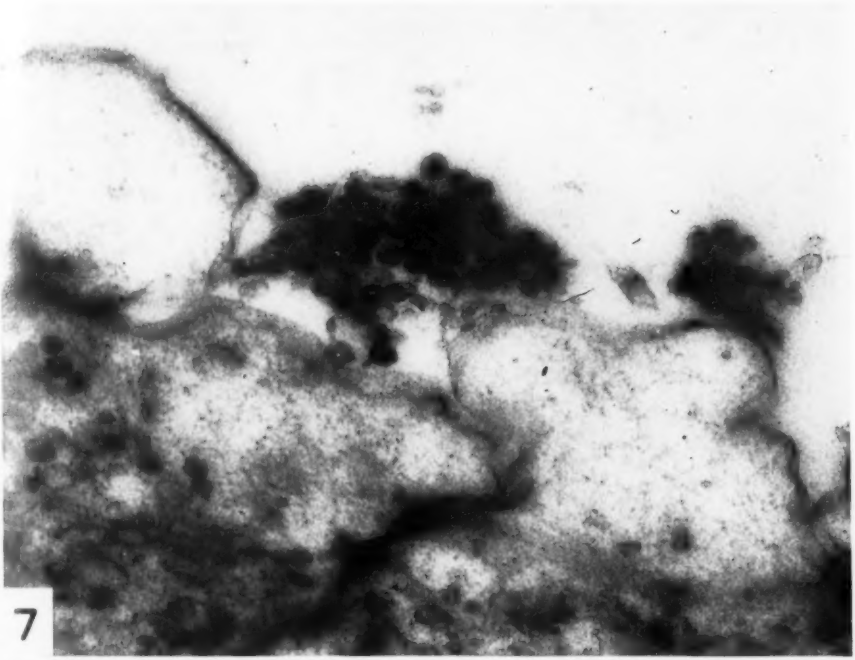
PLATE 109

FIGURE 7.—Parotid tumor induced in Swiss mouse by virus preparation. Figure shows aggregate of Type C particles close to surface of group of cells forming part of acinus. A few intracytoplasmic particles of less uniform size can be seen at *lower left*. Their connection, if any, with extracellular particles is uncertain. × 38,000

FIGURE 8.—Micrograph showing part of nucleus of cell from same parotid tumor as illustrated in figure 7. Within nucleus in two main groups (*upper left* and *lower right*) are scattered Type D particles. × 25,000



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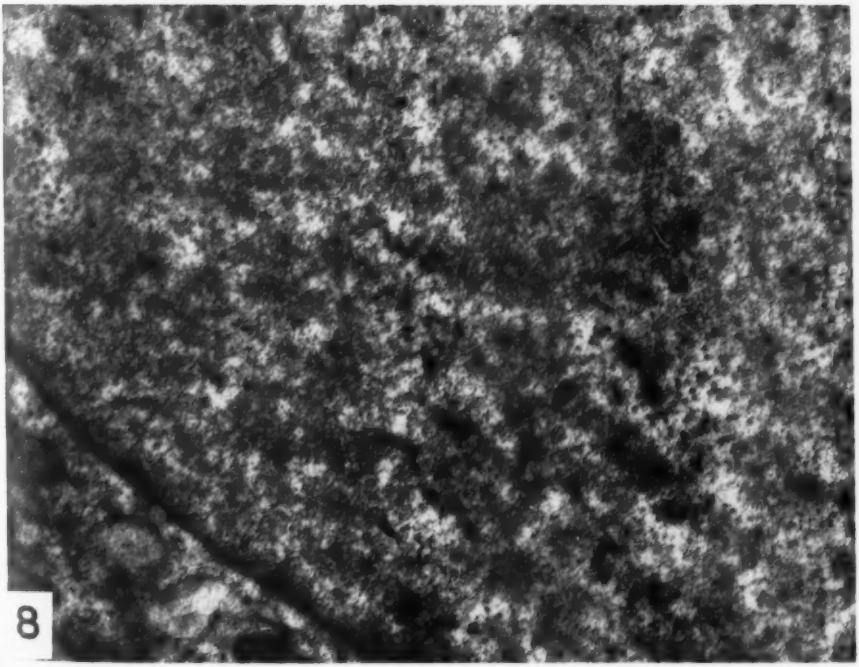

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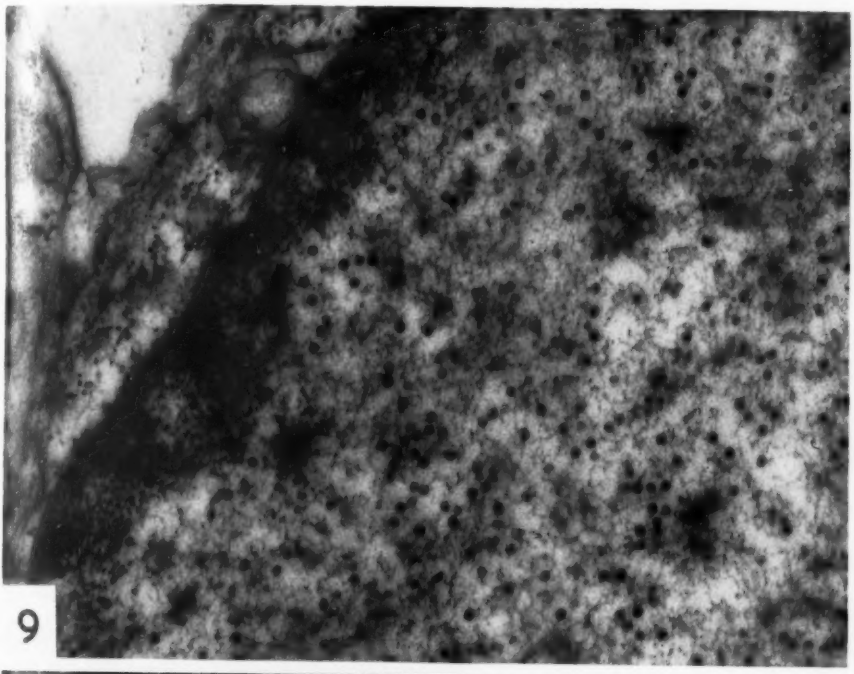
PLATE 110

FIGURE 9.—Stromal cell from hamster kidney 6 days after infection showing part of nucleus and small strip of cytoplasm at the *top left* corner. Throughout nucleus are scattered Type D particles. A rim of dense chromatin remains closely applied to nuclear membrane. × 48,000

FIGURE 10.—Array of closely packed Type D particles. Several distinct crystalline formations visible within aggregate. Similar crystalline arrangements have been seen within nuclei and phagocytosed inclusions of infected hamster kidney cells, and also in mouse kidney cells grown *in vitro* and infected with virus. × 48,000



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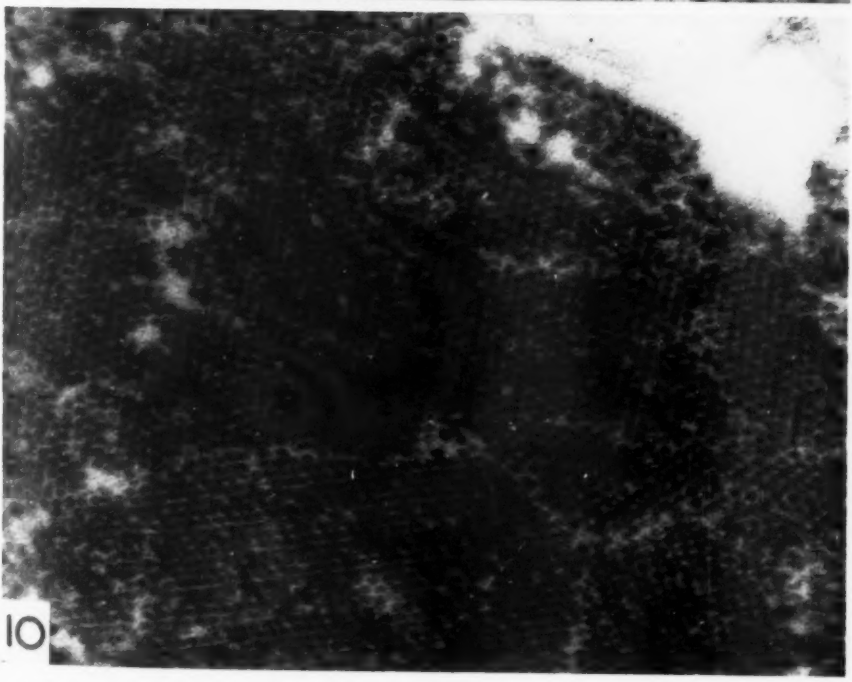

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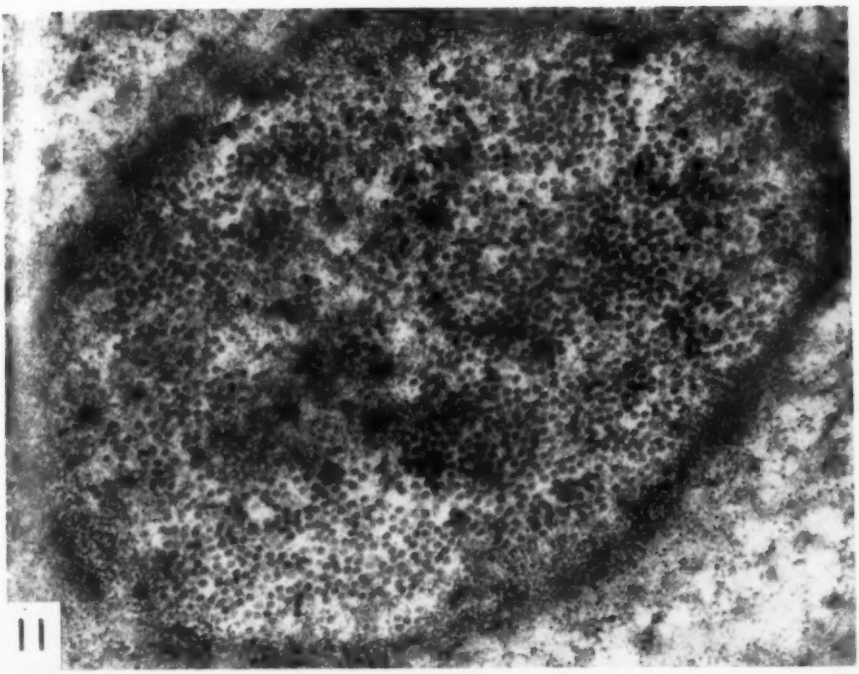
PLATE 111

FIGURE 11.—Mouse kidney cell from culture showing cytopathogenic effect after infection with virus preparation. Section has passed close to tip of nucleus resulting in an indistinct nuclear membrane. Most of nuclear substance has been replaced by closely packed Type D particles. × 38,000

FIGURE 12.—Part of cytoplasm of phagocytic cell in infected hamster kidney showing two inclusions containing closely packed particles. Most of these are Type D but in inclusion at *right* there are larger particles with a distinct limiting membrane and central nucleoid. × 46,000



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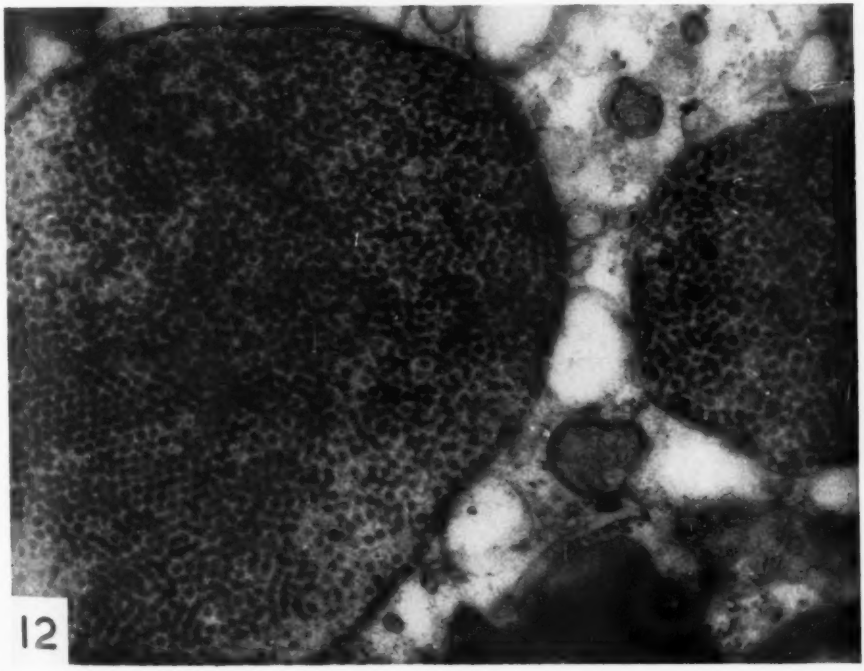

12

Studies on Eight Transplantable Plasma-Cell Neoplasms of Mice [1]

MICHAEL POTTER *and* JOHN L. FAHEY,[2] *National Cancer Institute,*[3] *Bethesda, Maryland*

SUMMARY

Eight plasma-cell neoplasms from mice of strain C3H/He or BALB/cAn, which were associated with the appearance of serum myeloma globulin in each new host, have been maintained in transplant. Each neoplasm was differentiated from the others by a characteristic serum- or urinary-protein electrophoretic pattern that developed during progressive growth of the transplanted tumor. The various plasma-cell neoplasms produced a variety of globulins with electrophoretic mobilities extending from the γ- to the α-region. Each neoplasm produced proteins that were confined to a part of this globulin region, which indicated a restricted protein-producing capacity. Two neoplasms, which produced similar serum globulin electrophoretic patterns, were differentiated by the appearance of Bence Jones protein in the urine of mice bearing one of the neoplasms but not in mice bearing the other. During serial transplantation, or transplantation to hybrids and certain other strains, the characteristic serum- and urinary-protein changes remained constant and did not change. It was concluded that the globulin production unique to each tumor was a stable heritable characteristic.—J. Nat. Cancer Inst. 24: 1153–1165, 1960.

DURING THE last 3 years we have obtained 8 plasma-cell neoplasms and carried them in transplant in inbred mice. Each neoplasm during progressive growth has produced in a new normal recipient one or more serum myeloma globulins, usually in great quantity, and has been associated with a serum- or urinary-protein electrophoretic pattern that differs from all of the others. The plasma-cell neoplasms were given to us by several investigators who discovered the original tumors in experimental mice and established them in transplant—some have already been described (*1–6*), others have not. Pertinent facts regarding the origin of the 8 tumors are included in table 1.

In this report a general account of our experience will be given and certain characteristics of the tumors described, the most notable being

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[2] The authors wish to thank Drs. H. Ira Pilgrim, Ruth M. Merwin, Katherine Sanford, and Thelma B. Dunn for the plasma-cell neoplasms from which our transplantation lines were begun (*see* table 1).

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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TABLE 1.—Source of 8 transplantable plasma-cell neoplasms

Neoplasm designation*	Original host			Established in transplant		Experimental procedure†
	Strain	Sex	Age (*mos.*)	Year	Investigator	
X5563	C3H/He CRGL	♀	22. 5	1955	Pilgrim	Ovariectomy—2
X5647	C3H/He CRGL	♀	22. 5	1955	Pilgrim	Ovariectomy—4
SPC-1	C3H/HeN	♂	12	1958	Sanford	425 r Whole-body X-ray—1; intramuscular injection C3H/He embryonic mince—1
DPC-1	C3H/HeN	♂	17. 5	1959	Dunn	On day of birth subcutaneous injection; whole AKR exorbital lacrimal gland
MPC-1	BALB/cAn	♀	11	1958	Merwin	Implant Millipore diffusion chamber containing C3H mammary-tumor tissue—subcutaneous diethylstilbestrol pellet
MPC-2	BALB/cAn	♀	14	1958	Merwin	
MPC-3	BALB/cAn	♀	14	1958	Merwin	
MPC-4	BALB/cAn	♀	17. 5	1959	Merwin	

*Recently we have adopted a system of designation in which 3 letters and a number are used: the first letter is the initial of the investigator; PC is plasma-cell neoplasm; and the digit is the tumor number in its order of appearance; *e.g.*, MPC-3 stands for Merwin plasma-cell neoplasm #3.

†Number indicates months of age when procedure was done.

the stability of the serum- and urinary-protein patterns throughout the transplantation history. The plasma-cell tumors have come from two main sources.

The first source of these tumors has been the ileocecal plasma-cell neoplasms found in strain C3H/He mice (*2*, *7*). Plasma-cell neoplasms may arise "spontaneously" in this strain, but usually animals that come from experiments designed for purposes other than inducing plasma-cell neoplasms were the ones in which plasma-cell neoplasms were found. For example, Dr. H. Ira Pilgrim was studying the effect of ovariectomy on the appearance of mammary tumors, and he found several large ileocecal tumors in old C3H/He CRGL experimental mice. He transplanted these tumors, and then realizing they might be similar to the plasma-cell neoplasms described by Dr. Thelma Dunn (*7*), of the National Cancer Institute, sent mice bearing these transplanted tumors, along with sections, to Dr. Dunn for confirmation. Two of the plasma-cell neoplasms in the present series came from this source X5563 and X5647. Dr. Katherine Sanford, of the National Cancer Institute, found an "unusual tumor" in the abdomen of a 1-year-old C3H/He male which had been inoculated intramuscularly with C3H/He embryonic tissue and irradiated with 425 r total-body radiation, at 1 month of age. Later the tumor was identified microscopically as a plasma-cell neoplasm; fortunately at this time Dr. Sanford had the tumor established in transplant. It has been designated SPC-1.

The second source of the plasma-cell neoplasms has been from the experiments of Dr. Ruth Merwin, of the National Cancer Institute (*4*, *5*). A group of BALB/cAn mice were implanted intraperitoneally with Millipore diffusion chambers containing C3H mammary-tumor tissue and subcutaneously with diethylstilbestrol pellets. Seven of 46 mice so treated developed bloody ascites with multiple neoplastic plasma-cell tumor nodules in the viscera and on the peritoneal surfaces. Four of these neoplasms have been established in transplant (MPC-1, 2, 3, 4).

Dr. Dunn has found plasma-cell neoplasms in the ileocecal area of C3H/He mice which had not been subjected to any experimental procedure (*8*). She has described several related changes in the ileocecal region of C3H/He mice, including: ulcers lined with plasma cells, ulcers with large collections of plasma cells (microscopic plasma-cell neoplasms), and finally large macroscopic plasma-cell tumors several centimeters in size (*2*, *7*, *8*). Recently a large ileocecal tumor mass was observed by Dr. Dunn in a 17½-month-old C3H/He mouse which had been inoculated at birth with AKR exorbital lacrimal gland (*8*). This lesion was photographed (fig. 1) and transplanted. Later it was identified microscopically as a plasma-cell neoplasm. It has been successfully transplanted (DPC-1).

Dr. Dunn has attempted to transplant many other lesions which appeared in the ileocecal area. Although some were tumors and most were shown microscopically to consist of abnormal plasma cells, only rarely has transplantation resulted in the establishment of a plasma-cell tumor line (*8*).

MATERIALS AND METHODS

Routine transplantation was done by the subcutaneous introduction of minced tumor tissue through a fully loaded 2½-inch, 12- or 13-gauge trocar (beveled thoracentesis needle, with smooth, rounded, obturator protruding 4–5 mm. beyond needle tip).

X5563, X5647, SPC-1, and DPC-1 were carried in C3Hf/Lw or C3H/He strain mice and the MPC series was carried in BALB/cAn. Both sexes were used interchangeably.

The plasma-cell neoplasm X5563 has not been established in all C3H recipients. Regression of an established tumor, however, has never been observed. Many mice, which have not developed tumors following a first inoculation, however, have been successfully inoculated at a later time. Failure to establish itself as a transplant in 100 percent of cases is a common feature of the C3H plasma-cell neoplasms. With the MPC group of plasma-cell neoplasms, a few BALB/c mice in early transfers failed to develop a progressively growing MPC-4 transplant. After the fifth-transfer generation, however, all mice have developed progressively growing tumors after inoculation. The plasma-cell neoplasms MPC-1, 2, and 3 have grown progressively in all BALB/c mice so far inoculated. Over 100 mice have been inoculated with each of these 3 neoplasms.

Hybrids of C3Hf/Lw were made by crossing C3Hf/Lw with NH, STOLI, DBAf/2, C57BR/cd, C57BL/Ka, and C58. The hybrids of BALB/cAn were made by crossing BALB/c with A/He and DBA/2. DBAf/2Lw strain mice were used in certain homologous transfers. C3Hf/Lw is closely related to both C3H/He, CRGL, and C3H/HEN—C3H/He CRGL and C3Hf/Lw are sublines of C3H/He. In contrast, C3Hf/Lw lacks the milk agent.

X5563, X5647, and SPC-1 require transplantation every 3 to 4 weeks. The MPC group of tumors grow more rapidly, and after the fifth-transfer generation all have been transplanted every 2 to 3 weeks. The subcutaneous tumor masses become very large and necrosis and skin ulceration occur. Transplantation of smaller tumors, before ulceration of the skin occurs, lessens the chance of bacterial infection.

The MPC group of tumors can be carried by serial intraperitoneal transfers. Although the tumors have produced an abundant ascites when carried this way, the neoplastic plasma-cell population has been sparse, usually less than 20 million tumor cells per mouse. The ascites tumor cells, however, are large and easily recognized in wet preparations. Small numbers of these cells establish a transplant in a new host.

Serum was obtained by cardiac puncture with a ¾-inch 23-gauge needle, from mice anesthetized with ether. The needle was introduced slightly to the left of the xiphoid and passed through the abdominal wall and diaphragm. Mice bearing tumors 2 cm. or more in diameter readily yield between 1 and 2 ml. of whole blood by this method.

Urine was obtained directly by placing a test tube at the urethral orifice. Slight pressure on the bladder—often readily palpable—with the

tip of the finger will induce micturition. Female mice were used for these collections as the bladder is more readily palpated. Urine so collected is free of feces. Samples were collected 2 or 3 times daily from tumor-bearing mice.

All of the electrophoretic studies were done with the Spinco paper electrophoretic apparatus. The methods were previously described (*1*).

RESULTS

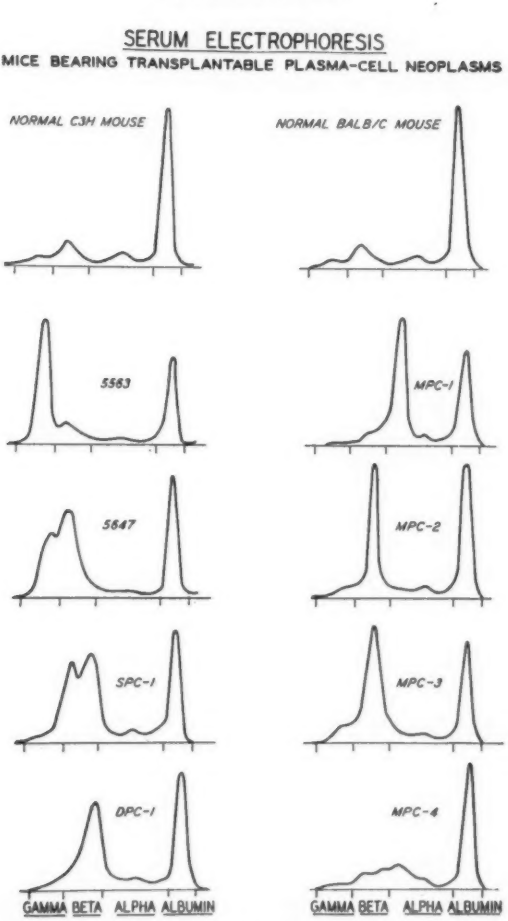
Serum and Urine Protein Changes Associated With the Plasma-Cell Neoplasms

The characteristic serum and urine electrophoretic patterns of normal mice of strains C3Hf/Lw and BALB/cAn have been compared with the serum and urine patterns found in mice bearing the various plasma-cell neoplasms. The plasma-cell neoplasms were placed in 2 groups, according to strain of origin. Each of the 4 C3H plasma-cell neoplasms produced myeloma proteins of different electrophoretic mobilities. X5563 was characterized by a single discrete peak in the γ-globulin region (*1*); X5647 by a broader peak in the slow β-globulin region (*3*); SPC-1 by a peak in the fast β-region; and DPC-1 by a peak in the α-3 region. The 4 BALB/c neoplasms also showed serum electrophoretic patterns with myeloma globulins of different mobilities (text-fig. 1). MPC-1 showed a single α-3-globulin mobility peak, MPC-2 and 3, fast β-globulin mobility peaks, and MPC-4, 2 small peaks in the α-3 mobility region.

The serum electrophoretic changes noted in the foregoing are characteristically associated with plasma-cell neoplasms. Since 1957 we have examined 95 serums taken from mice with "spontaneous" and induced neoplasms and from mice bearing transplantable reticular neoplasms, but no increase in serum globulins has been found. Included in this group were 2 serums obtained from mice bearing sarcomas that arose in BALB/c mice implanted with Millipore diffusion chambers. Neither was associated with increases in serum globulin in mice carrying transplants.

The quantitative as well as the qualitative serum-protein changes produced by each tumor differed. The alterations in total serum protein, the percent of the myeloma globulin, and the quantity of the individual constituents are given in table 2. Each value represents the average of 5 determinations from mice bearing 5 to 8 gm. subcutaneous tumors, without respect to the post-transplantation or transfer generation. It may be seen from this table that each of the neoplasms elevates the total serum protein from the normal value of 5.4 gm. percent to average levels as high as 9.8 gm. percent. This elevation is accounted for by the myeloma globulin. Other constituents of the serum protein remain unchanged or decreased in amount. MPC-4 produced the least amount of myeloma globulin and stands out from the rest of the group in this respect.

Urine collected from mice bearing the various transplanted tumors (except DPC-1) has been concentrated by ultrafiltration and then sub-



TEXT-FIGURE 1.—Paper electrophoretic analysis of serum from normal and various plasma-cell neoplasm-bearing mice: Analytol tracings. (Spinco Division, Beckman Instruments, Inc., Belmont, Calif.)

jected to paper electrophoresis. Mice normally excrete a number of proteins, of various mobilities, in the urine in very small amounts. In only one case was a large amount of protein in the urine detected and that was in mice bearing MPC-2, the plasma-cell neoplasm. A previous publication (*6*) has already reported that this protein has the characteristics of a Bence Jones protein with the electrophoretic mobility of a γ-globulin, precipitating in acidified urine when heated to about 50° C., returning to solution at boiling, and reappearing on cooling.

The urinary protein had a sedimentation coefficient in the ultracentrifuge of 3.4S ($S_{20,w}$) while the serum myeloma globulin was about 6.5S. It may be seen that in the BALB/c group, mice bearing plasma-cell

TABLE 2.—Quantitative alterations in serum proteins in mice bearing the 8 plasma-cell neoplasms*

Neoplasm	Serum protein in gm. percent							Percent myeloma globulin
	Total	Albumin	Globulins					
			α_1	α_2	α_3	β	γ	
Normal	5. 4	2. 95	0. 25	0. 48	0. 36	0. 87	0. 46	—
X5563	9. 8	1. 85	0. 23	0. 43	0. 42	0. 86	6. 06	61. 6
X5647	7. 7	2. 72	0. 25	0. 50	0. 46	2. 16	1. 50	52. 2
MPC-2	9. 2	2. 32	0. 20	0. 37	0. 49	4. 99	0. 82	54. 2
MPC-3	7. 3	2. 09	0. 20	0. 42	0. 37	3. 44	0. 80	47. 1
SPC-1	7. 6	2. 36	0. 24	0. 31	0. 32	4. 08	0. 56	53. 6
MPC-4	5. 9	2. 57	0. 17	0. 42	1. 04	1. 28	0. 27	30. 4
MPC-1	8. 4	2. 29	0. 19	0. 33	4. 73	0. 54	0. 31	56. 4
DPC-1	7. 5	2. 45	. 20	. 29	3. 08	1. 30	. 20	41. 8

*The values given in this table represent the averages obtained from 5 mice bearing 5 to 8 gm. tumors, with the exception of those values given for SPC-1, which were averages of 5 mice bearing 3 to 5 gm. tumors, and those given for DPC-1, which were based on a single determination.

neoplasms MPC-2 and 3 share a similar serum electrophoretic pattern, single β-globulin peaks, but only one is associated with Bence Jones proteinuria. Mice bearing MPC-2 also develop a pathologic lesion in the kidneys characterized by extensive large and small tubular casts.

Effect of Transplantation in the Same Strain

The serum and urine electrophoretic patterns associated with each of these neoplasms has been followed during serial transplantation (table 3). The individual characteristic pattern has remained the same in every case examined. The number of transfer generations and the number of individual serums examined are given in table 3. X5563 has been transferred 25 times and during 3½ years no detectable change in the electrophoretic characteristics of the serum myeloma globulin has been observed. Although the other tumor lines have been transferred fewer times—DPC-1 was represented, so far, by only the original mouse and first-generation transfer samples—the serum-protein patterns have also remained constant.

Serums were first examined during first- or second-transfer generation of all these tumors except DPC-1 (table 3). Serum from the mouse bear-

TABLE 3.—Transplantation characteristics of 8 plasma-cell neoplasms

Neoplasm	Mobility of myeloma globulin	Transfer generation		Number of individual serum samples examined by electrophoresis
		First studied	Latest determination	
X5563	γ	2	24	99
X5647	β (slow)	2	13	87
SPC-1	β (fast)	2	6	13
DPC-1	α-3 (slow)	original host	1	2
MPC-1	α-3 (fast)	1	12	61
MPC-2	β	1	10	58
MPC-3	β	1	11	11
MPC-4	α-3 (two)	1	8	30

ing the original DPC-1 tumor (fig. 1) was available for study. Although it has been assumed, for the other neoplasms, that the changes associated with the transplanted tumors are similar to those in the original host, the finding of identical serum-protein changes in the original and first-transplant generation of DPC-1 proved the constancy of this characteristic for neoplasm DPC-1 and supports the assumption of a constant relationship for the other plasma-cell neoplasms.

Dr. Merwin transplanted 4 different first-transfer generation lines from the original MPC-2 mouse—3 were taken from separated peritoneal nodules and one from the hemorrhagic ascites. All 4 produced the same serum electrophoretic patterns.

MPC-1, which has been carried by serial transfer of ascites, was introduced into mice, with graded doses of cells. Five of 5 mice inoculated with 100 cells developed a progressively growing tumor and one in 5 mice inoculated with 10 cells developed tumor ascites. A transfer line was initiated from the mouse that developed ascites from the 10-cell inoculum, and after 5 serial transfers mice bearing this line of MPC-1 had the characteristic peak of globulin concentration in the α-3 region in both serum and ascites.

Effects of Transplantation to Hybrids and Homologous Strains

Different transplantation situations were examined to determine whether changing the host would influence the characteristic serum- or urinary-protein patterns. First-generation (F_1) hybrids were used largely for this purpose. Such hybrids would be expected to allow tumor growth but also to present a different host environment to the growing tumor. The BALB/c tumors grew just as well in hybrids as they did in the inbred host. In most cases the C3H neoplasms did as well if not better in hybrids. One exception was noted in a cross between NH and C3Hf/Lw. X5563 did not establish itself well in this F_1 hybrid and there was a striking reduction in positive mice compared with C3Hf/Lw and other F_1 hybrids. This point is being further investigated.

It was found that X5563 produced the same characteristic electrophoretic pattern with a discrete γ-globulin mobility peak in 4 different F_1 hybrids of C3Hf/Lw (STOLI, C58, C57BL/Ka, and DBAf/2).

With X5647, the ultracentrifugal as well as the electrophoretic characteristics of the serum proteins were investigated in hybrid transplantation studies and included 2 F_1 hybrids of C3Hf/Lw: DBAf/2 and C57Br/cd. The myeloma globulins associated with X5647 appear in the ultracentrifuge as 3 components with sedimentation coefficients ($S_{20,w}$) of 9, 11, and 13S. The characteristic ultracentrifugal pattern including components of 9, 11, and 13S as well as the characteristic electrophoretic patterns were found in each hybrid studied.

Bence Jones protein production was examined by transplanting MPC-2 into female (BALB/c $\times$ DBA/2)F_1 hybrids. The electrophoretic mobility and solubility characteristics of the urine proteins were tested and found to be unchanged. The 4 BALB/cAn plasma-cell neoplasms will

grow progressively in some individuals of strain DBAf/2. MPC-1 grew progressively in 6 of 8 DBAf/2 MPC-2 in 2 of 16, MPC-4 in 1 of 8 DBAf/2. MPC-3 grew progressively in 6 of 8 DBAf/2 and has been carried for 3 successive transfers in this strain. MPC-3 and MPC-1 have not grown in C3Hf/Lw, and MPC-3 has failed to grow in strain C58.

DISCUSSION

The remarkable features of the transplantable plasma-cell neoplasms in mice were: (1) The group of neoplasms produced a number of globulins as evidenced by abnormal peaks of globulin concentration with different electrophoretic mobilities. (2) No single neoplasm produced all of these globulins, each was restricted to making globulins which appeared within a part of the globulin region on the paper electrophoretic strip. (3) The characteristic serum electrophoretic pattern associated with each neoplasm did not change during transplantation. We concluded from these findings that the characteristic globulin production of a plasma-cell neoplasm was stable and heritable.

Although our experience to date has concerned only 8 plasma-cell neoplasms, we have been able to differentiate by the paper electrophoretic patterns of serum and urine any one from all the others regardless of whether the 1 neoplasm was compared with the 3 others found in the same strain or all 7 others.

How are these individual characteristics established biologically? Individuality may be the result of the carcinogenic transformation of the plasma cell. In view of the stable heritability of the globulin production in the neoplastic population, the effects of carcinogenesis must involve the genetic apparatus of the cell. A random mutation, for example, whose effects are phenotypically expressed in the globulin-synthesizing apparatus could be postulated.

A more plausible origin of the individuality of myeloma-globulin synthesis may stem from a specialization established in normal plasma cells before carcinogenesis. The plasma-cell population would be composed of multiple fixed cell types or clones. Carcinogenesis begins in and involves only one of these clones.

A division of labor in normal antibody-forming cells is strongly supported by experimental evidence from several sources (*9–11*). Nossal and Lederberg demonstrated with single cells isolated from lymph nodes of rats immunized with 2 salmonella serotypes that some isolated single cells could immobilize mobile salmonella by liberating antiflagellar antibody. When such a cell was observed to immobilize one of the salmonella types, it did not immobilize the other. Nossal has subsequently shown, histologically, that the antibody-forming cells are almost entirely plasma cells or plasma blasts (*12*).

This apparent division of labor in antibody-forming cells later prompted Lederberg to propose the following on the subject of antibody-forming

cells: "Mature cells proliferate extensively under antigenic stimulation but are genetically stable and therefore generate large clones genotypically pre-adapted to produce the homologous antibody" (*13*). While a division of labor in normal plasma cells may serve to explain how cells produce antibody, it cannot be directly transposed to explain individual globulin characteristics seen in the transplantable plasma-cell neoplasms. The division of labor in the neoplasms relates to differences in electrophoretic mobility of globulins, not specific antibody production.

While these studies mimic the experience with multiple myeloma in man regarding variation in the myeloma globulin from patient to patient (*14*), they do add 2 additional points. First, myeloma globulins can vary in electrophoretic mobility even when they are obtained from plasma-cell neoplasms that developed in members of the same inbred strain. Second, by transplantation the population of neoplastic cells can be grown in many different hosts and over an extensive period of time, and even then, the myeloma globulins remain the same for each neoplasm.

Rask-Nielsen *et al.* (*15*) and Clausen *et al.* (*16*), working with 2 transplantable plasma-cell leukemias, have found differences by using the technique of immunoelectrophoresis. Cobau and Korst (*17*) have reported that X5563 produces a characteristic γ-mobility-protein when grown in Swiss mice.

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PLATE 112

FIGURE 1.—A 17½-month-old C3H/HeN male with original ileocecal plasma-cell neoplasm (*lower arrow on left*) and mediastinal metastases (*upper arrow on right*). *Note also* hepatoma found in this mouse (*lower arrow on right*). Wall of the cecum is infiltrated and thickened; mesenteric node visible between the liver and ileocecal mass is greatly enlarged. This is characteristic appearance of primary plasma-cell neoplasms in strain C3H mice. This neoplasm (designated DPC-1) was successfully established in transplant by transplanting tissue from ileocecal mass and mesenteric node.

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Field Trial of the Cytoanalyzer: 1,184 Specimens Analyzed [1, 2]

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SUMMARY

After much research and development the Cytoanalyzer was subjected to its first clinical trial. In this preliminary attempt to prescreen electronically specially prepared uterovaginal specimens, 1,184 specimens were used including 1,163 negatives and 21 from patients with uterine cervical malignancy. The lowest value obtained from any positive smear was established as an arbitrary base-line value of abnormal cells per smear. With this value, 36.6 percent of the smears scanned could have been eliminated as negatives without cytotechnical screening. The results of this trial are encouraging and indicate that automatic electronic prescreening of smears can be an aid to the field of exfoliative cytology.—J. Nat. Cancer Inst. 24: 1167–1179, 1960.

THE FIRST clinical trial of the Cytoanalyzer,[7] type 401, was a preliminary attempt to prescreen uterovaginal specimens electronically. The machine was designed to aid the field of exfoliative cytology by separating smears into the two broad categories—"negative" and "questionable." Since normal cellular elements can be distinguished from those that are abnormal on the basis of two criteria, namely, nuclear area and nuclear optical density, the machine measured the smears by these two criteria and then, on the basis of the relative population of abnormal cellular elements, placed each smear in its proper category. The "negatives" consisted entirely of smears that would be so classified

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[6] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[7] "Cytoanalyzer" (*1*) is the designated term for an advanced experimental computing instrument, designed by the Department of Medical and Biological Physics, Airborne Instruments Laboratory, Division of Cutler Hammer, Mineola, N.Y.

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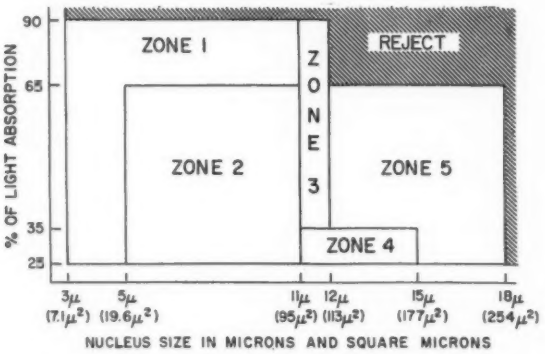
by a cytotechnician or pathologist. All positive and suspicious smears as well as some negatives that required clinical evaluation and diagnosis were "questionable." Since uterovaginal material was most readily accessible and thoroughly studied cytologically, it was adapted by special techniques for use in this study.

The Cytoanalyzer uses parameters of mathematical relationships to separate cells into zones whose boundaries are arbitrary; they are based upon predetermined measurements of nuclear area and density (*2,3*), which may be used to indicate relative occurrences of different cell types (text-fig. 1).



TEXT-FIGURE 1.—Parameters of size and density.

Model cell types, when mathematical definitions are used, place most of the leukocytes in zone 1, most of the normal epithelial cells in zone 2, and most of the abnormal cells in zones 3, 4, and 5. While all cells in zones 3, 4, and 5 are considered abnormal, for purposes of evaluation by the machine they have been recorded in the separate zones. Zone 3 contains cells that are slightly larger than normal; zone 4 contains those that are larger than normal but are lightly stained; zone 5 contains those that are larger than normal and darkly stained. These zones are electronic designations and have no relationship to Papanicolaou classification of smears. Almost all smears will have a few elements in zones 3, 4, and 5. By computing the ratio of elements in zones 3, 4, and 5 (abnormal cells) to those in zone 2 (normal cells), we derive a numerical ratio of abnormal cells per smear, the size of which determines whether a smear is "negative" or "questionable."

The machine scans a smear on a slide moving back and forth automatically in a movable stage beneath a fixed microscope. After each longitudinal excursion is completed, the stage moves transversely one electronically defined field and then returns in a path parallel to the preceding longitudinal one. This procedure allows an orderly, comprehensive scanning of smears, similar to the method used by many cytotechnicians.

Certain rules of selection have been incorporated into the Cytoanalyzer. In a smear, all particles larger than 7.1 μ^2 that have a light absorption of more than 25 percent are evaluated. Smaller or less dense particles are ignored. Since there is no feasible way to eliminate all debris, overlapping cells, and clumps of cells from the smear, these must be recognized and rejected. The debris or groups of cells not characteristic of individual cells are counted in the reject zone. Particles are rejected that (*a*) absorb more than 90 percent of the light, (*b*) are larger than 20 μ in the transverse direction, (*c*) are larger than 18 μ in the longitudinal direction, (*d*) have cytoplasm absorbing more than 10 percent of the light, and/or (*e*) do not meet the shape requirements of a nucleus. Since the machine has difficulty resolving particles less than 2 μ apart, these are occasionally evaluated as one particle and may be recorded erroneously in zone 3, 4, or 5, but they are usually eliminated by one of the rules of selection. The particles along the edge of the field are ignored (*4*) because complete measurements are not available. All the other cells are classified in zones 1 to 5, according to the combined measurements of nuclear area and density.

METHOD

The first clinical trial utilized 1,184 specimens prepared by the sieve-and-spray technique and stained by the Feulgen reaction (*5*). All specimens were collected, processed, and screened in the same manner. For each smear prepared for the Cytoanalyzer another was prepared by standard technique at the same time and from the same vaginal pool. A specimen was aspirated into a vaginal pipette and a portion was used to make a standard smear (*6*). For Cytoanalyzer samples the remainder of the specimen was washed into 5 ml. of normal saline. (Infrequently it was necessary to reaspirate from the vaginal pool to obtain a fully satisfactory specimen.) Immediately after the saline suspension was shaken to free the cells from the mucus, 45 cc. of absolute alcohol was added; the specimens were then refrigerated and shipped to the Cytoanalyzer Laboratory for processing.

The standard smears were processed and screened in laboratories independent of the Cytoanalyzer Project.[8] They were handled as routine smears (*6*); further clinical study and treatment were performed when indicated.

Specimens to be prescreened by the Cytoanalyzer were processed in the Cytoanalyzer Laboratory. The smears were prepared by the sieve-and-spray technique and stained with Schiff's reagent in the Feulgen reaction. This technique is stoichiometric for deoxyribonucleic acid (DNA) and produces a constant stain; it provides a moderate nuclear stain with minimal staining of the cytoplasm. Since the Cytoanalyzer

[8] The Washington Cytology Unit, Washington, D.C., Johns Hopkins Hospital, Baltimore, Md., and Walter Reed Army Hospital, Washington, D.C.

prescreens smears on the basis of nuclear area and optical density, the characteristics of the smear were adequate for evaluation.

RESULTS

The age, race, and marital status of participants in the clinical trial were similar to those of any random distribution group. All but 76 specimens were collected in sequential order from a general population. The 76 were collected from patients with suspicious cytological changes at previous examinations. At the time these specimens were collected for the clinical trial, 20 had definite malignant changes; specimens from this group were collected before any treatment was instituted. Nineteen patients had positive biopsies, but the other patient was unavailable for a biopsy. In one other instance in the general-population group, malignant changes were detected and proved by biopsy, which gave a total of 1,163 negative and 21 positive cases for evaluation.

The "ratio of abnormality" per smear was calculated for each slide by dividing the zone 2 entries into the total entries for zones 3, 4, and 5. The lowest "ratio of abnormality" for any positive smear was 0.391. With this value, an arbitrary separation point was established to divide the general-population smears into the "negative" and "questionable" groups. This criterion allowed 425 of the specimens, or 36.6 percent, to be classified as "negative" and 738, or 63.4 percent as "questionable." Thus, without risk of missing any of the actually suspicious or positive specimens, 36.6 percent of the smears would not have required screening by technicians.

The results of this trial are encouraging; they indicate that smears can be sorted reliably and consistently by an electronic scanning instrument. Manual measurements of nuclear size and density established that smears from cancer patients contain a significantly higher percentage of enlarged, hyperchromatic nuclei, which can be used as a criterion to sort smears. Atypical cells were found to comprise more than 0.5 percent of the total epithelial cells of cancer cases in one study (*3*). This high percentage of abnormal cells per smear is not necessarily indicative of malignant changes in the affected cells but is indicative of abnormal smear characteristics and warrants careful screening by cytotechnicians. As with all cytology examinations, a final diagnosis of malignancy, confirmed by adequate clinical and biopsy study, must be made by a qualified cytologist or pathologist.

CAUSE OF ERRORS

All satisfactory smears, *i.e.*, those with an adequate cell population, have some elements that are recorded in the abnormal cell zones. However, when elements other than single, enlarged, hyperchromatic nuclei are so recorded, errors result. These occur for various reasons. Ele-

ments that have certain configurations or are slightly out of focus may escape the machine's reject rules and be registered as abnormal cells (figs. 1 through 6).

Overlapping epithelial-cell nuclei account for 65 percent of the errors. There are many degrees of nuclear overlap, but only those conditions that create an area larger than 95 μ^2 and permit a proper amount of light absorption are recorded as abnormal. Many overlapping nuclei do not fulfill these criteria.

Leukocytes lying on top of epithelial-cell nuclei cause 10 percent of the errors. This is an uncommon error because leukocyte nuclei seldom exceed an area of 37.5 μ^2; thus, rather precise positioning of several nuclei would be necessary to simulate one abnormal nucleus.

Clumps of leukocytes cause up to 10 percent of the errors in a slide. Leukocytes tend to clump and occasionally assume a configuration that simulates a single enlarged nucleus.

Precipitation of dye and coagulation in the cytoplasm causes up to 10 percent of the errors. Because of the distribution and shape of these deposits, most of them are readily rejected.

Debris on the slide causes less than 5 percent of the errors because it is relatively easily recognized and rejected by the rules of selection.

The percentage and type distribution of errors varies with every slide. This problem requires further study and evaluation to determine its exact effect on the automatic scanning of smears.

DISCUSSION

The present Cytoanalyzer is an experimental, semiautomatic instrument. Placement, focusing, and removal of slides are done manually. Scanning, however, is automatic once a slide is inserted and in focus. These operations now take 15 minutes, after which notation of cell counts on permanent records and calculation of the "ratio of abnormality" are done by technicians. Future plans include development of automatic loading, focusing, and removal devices, and recording instruments to transcribe the desired information. These improvements will enable the machine to operate automatically for long periods without constant supervision. Along with these improvements, the scanning speed of the Cytoanalyzer can be increased by a device that will permit slides to be scanned faster than one per minute.

All smears prescreened by the Cytoanalyzer can be divided into two groups. The "negative" group will not need to be rescreened, whereas all smears in the "questionable" group will require screening by cytotechnicians. Smears stained by the Feulgen reaction can be screened by cytotechnicians who have had previous experience with this preparation. If smears need to be made more adaptable to screening by the technician, various counterstains can be used for the cytoplasm. Appropriate filters could be added to the optical system of the Cytoanalyzer to minimize the

effects of these counterstains when the smears are automatically scanned. In the first clinical trial, 36.6 percent of the smears were eliminated as negative by automatic, electronic prescreening, leaving 63.4 percent to be examined by cytotechnicians and pathologists. A machine that eliminates such a large percentage of specimens from the work load of a laboratory processing large numbers of specimens (*7*) would be a very valuable tool.

It is important to remember that a small number of positive specimens were available for this preliminary effort. The "ratio of abnormality" used to separate the "negative" from the "questionable" specimens may well change slightly as larger numbers of smears are scanned, technical improvements are made in the machine, and improvements are made in the smear. Some errors made by the machine can be eliminated by a self-focusing device designed to keep the cells in the best possible focus. The present selection rules could then be used to classify cells more critically and additional rules could be used to evaluate cellular elements. Thus, the zone counts would be more restrictive, which would refine the results of prescreening by the Cytoanalyzer.

The experience gained from the first trial emphasizes the need for improvements. With correction of the noted deficiencies and further study of other problems, it is hoped that improved and more significant results will be obtained in the next field trial.

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PLATES

PLATE 113

FIGURE 1.—Malignant epithelial-cell nucleus. Enlarged and hyperchromatic. Feulgen stain. × 775

FIGURE 2.—Isolated malignant epithelial-cell nucleus with normal size and shape. Feulgen stain. × 775

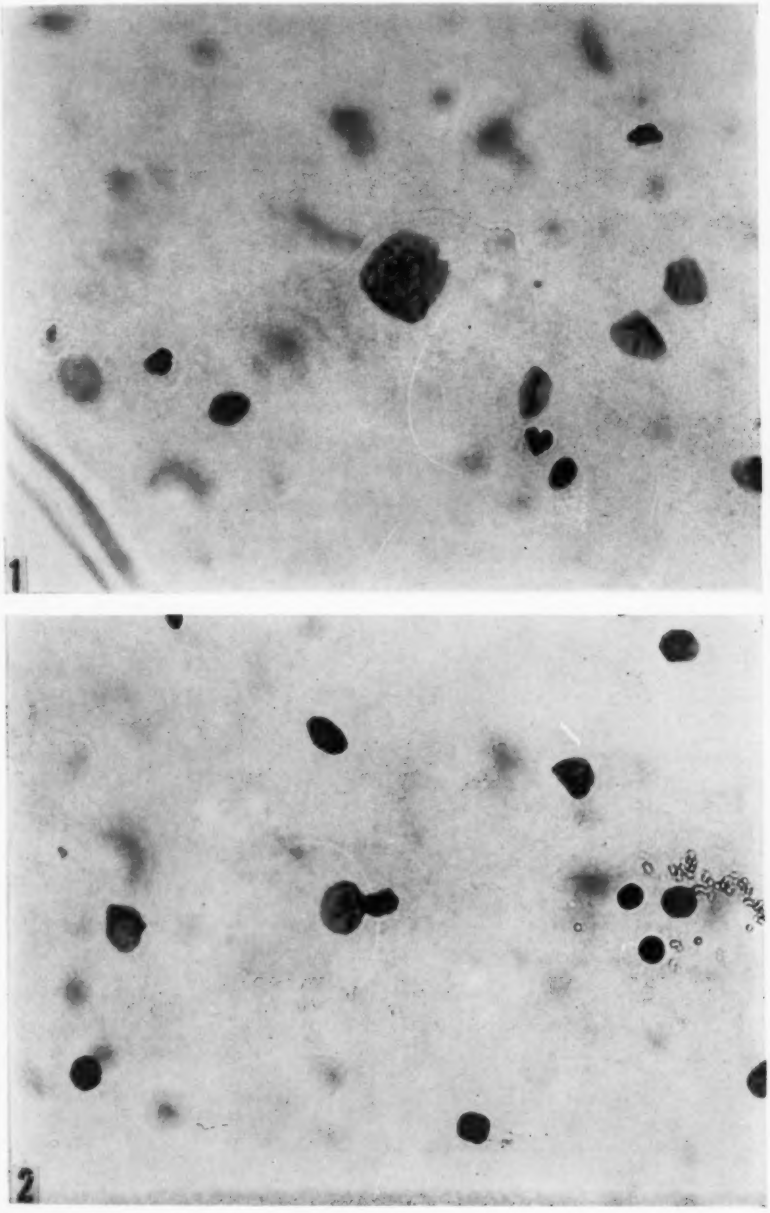
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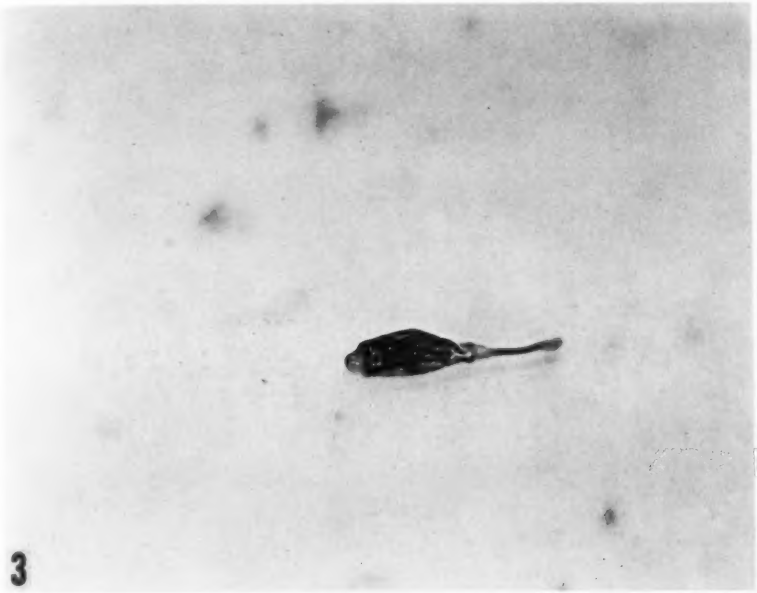
PLATE 114

FIGURE 3.—Two adjacent epithelial-cell nuclei. Combined area not large enough to be considered as one single abnormal nucleus. Feulgen stain. × 775

FIGURE 4.—Error. Cellular debris resembling size and shape of an abnormal nucleus. Feulgen stain. × 775



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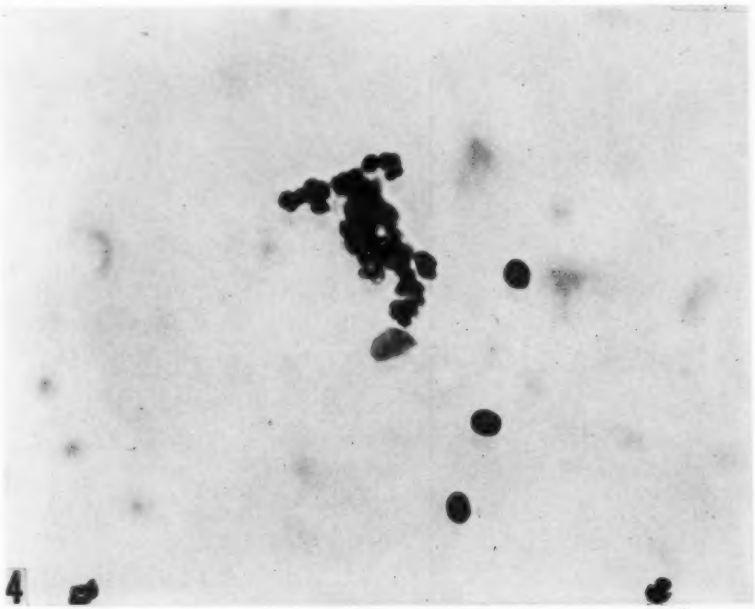

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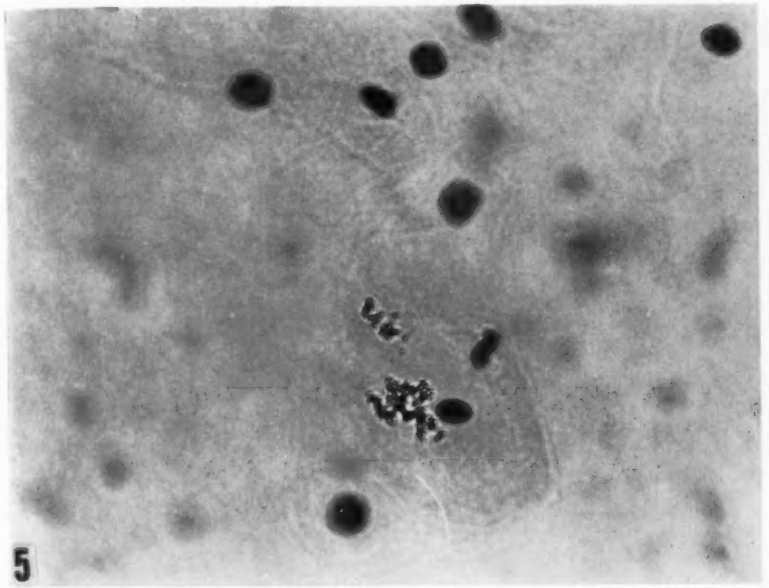
537982—60——23

PLATE 115

FIGURE 5.—Reject. Cluster of leukocytes not filling shape requirements of a single abnormal nucleus. Feulgen stain. × 775

FIGURE 6.—Reject. Crystallization of dye or coagulation of cytoplasm. Usually rejected on basis of shape or size. Feulgen stain. × 775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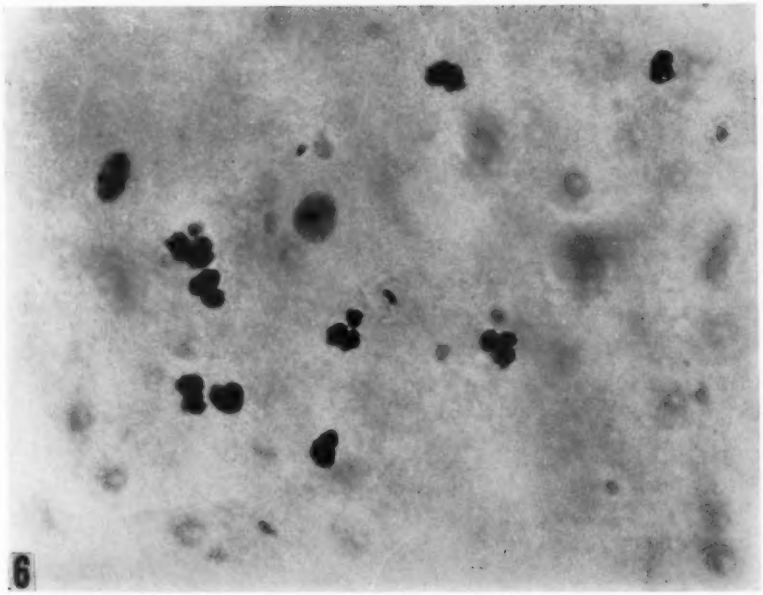
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Pituitary Growth Hormone and Seromucoid [1, 2]

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SUMMARY

The seromucoid of rats with intact pituitary glands was increased only slightly by the administration of large amounts of bovine pituitary growth hormone. In hypophysectomized rats the serum mucoproteins were significantly lower than in intact rats. Hypophysectomized rats responded to daily injections of growth hormone, in amounts as low as 0.025 mg., by an increase in the seromucoid levels. The increase in seromucoid was proportional to the dose of pituitary growth hormone.—J. Nat. Cancer Inst. 24: 1181–1185, 1960.

PITUITARY GROWTH hormone is important in the regulation of protein and carbohydrate metabolism (*1–4*), in carcinogenesis (*5*, *6*), and in inflammation (*7*). Since seromucoid, *i.e.*, serum mucoprotein, is markedly elevated in neoplastic and inflammatory states (*8–10*), it seemed likely that pituitary growth hormone was concerned in its metabolism. The present studies were undertaken to ascertain the effects of hypophysectomy and of pituitary growth hormone on seromucoid levels in rats.

MATERIALS AND METHODS

Young female rats of the Long-Evans strain, 21 to 25 days old, were selected. In the first of the two series of experiments, animals with intact pituitary glands were observed, and in the second, hypophysectomized animals were used. All animals were maintained on a stock diet—Rockland complete rat diet. The rats were weighed at the beginning of the study and every 5 days thereafter for 20 days.

Normal rats.—Six rats were maintained for the first 10 days without treatment. During the final 10 days of the experiment each was injected daily, intraperitoneally, with 5.25 mg. of bovine pituitary growth hor-

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[3] The author wishes to thank Dr. C. H. Li for the bovine pituitary growth hormone and to acknowledge the able technical assistance of Mrs. Carolyn Ellis.

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mone (*11*). The controls were maintained without treatment for 20 days. All animals were fasted for 24 hours before they were killed.

Hypophysectomized rats.—The rats were hypophysectomized at 21 to 25 days of age and observed for 10 days without treatment to determine the completeness of hypophysectomy. Animals that showed a significant increase in weight were discarded. Five groups of 10 rats each were selected for the study. They were injected intraperitoneally with purified bovine pituitary growth hormone daily for 10 days. The amounts given daily ranged from 0.025 to 2.0 mg. Sixteen hypophysectomized rats were maintained without treatment as controls. Food was also withheld from these animals for 24 hours before they were killed.

Before the rats were killed, they were anesthetized by intraperitoneal injections of sodium pentobarbital (Nembutal). Blood was obtained by cardiac puncture for determination of the seromucoid content. The sellae turcicae of the hypophysectomized rats were examined under a dissecting binocular microscope; fragments of tissue in the sellae were studied histologically. Incompletely hypophysectomized rats were excluded from the series.

The seromucoid of duplicate samples from the same animal was determined by means of a modified Winzler technique (*12*). A filtrate was prepared after precipitation of serum proteins by perchloric acid; the seromucoid fraction was obtained from the filtrate by precipitation with phosphotungstic acid. The precipitate was then dissolved in a solution of sodium carbonate, Folin-Ciocalteau phenol reagent was added to the solution, and the tyrosine content was determined spectrophotometrically at 600 $\mu\mu$.

RESULTS

Normal Rats

The control rats with intact pituitary glands grew at a normal rate; during the final 10 days of the study the average gain in weight among the controls was 32.8 gm. (table 1). The rats injected with pituitary growth hormone grew more rapidly; these animals gained an average of 47.6 gm. during the experiment.

The seromucoid level of the control rats in this group was 2.25 ± 0.024 mg. percent tyrosine. In the group injected with growth hormone the concentration of seromucoid was slightly but significantly elevated, 2.60 ± 0.127 mg. percent tyrosine.

Hypophysectomized Rats

The average weight gain of the controls in the hypophysectomized series was 1.4 gm. during the experimental period of 10 days (table 2). In the group of hypophysectomized rats injected with 0.25 mg. of growth hormone the average increase in weight was 16.0 gm. during the same period.

TABLE 1.—Effect of pituitary growth hormone on seromucoid of rats with pituitary glands

Group	Growth hormone total dose (*mg.*)	Period of injection (*days*)	Number of rats	Age at autopsy (*days*)	Average weight at autopsy (*gm.*)	Gain* (*gm.*)	Seromucoid mg. percent tyrosine (*S.E.*)
A Control	0	0	18	45–55	129. 0	32. 8	2. 25 (±0. 024)
B Experimental	52. 5	10	6	42	164. 8	47. 6	2. 60 (±0. 127)

*Increase in weight during final 10 days of experiment.

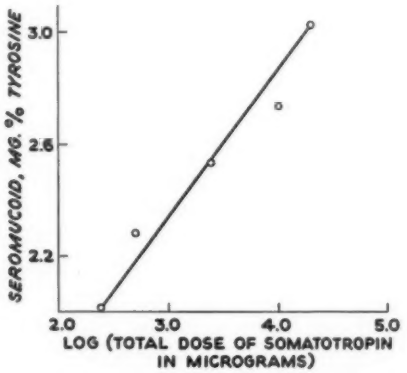
TABLE 2.—Effect of pituitary growth hormone on seromucoid of hypophysectomized rats

Group	Growth hormone total dose (*mg.*)	Period of injection (*days*)	Number of rats	Age at autopsy (*days*)	Weight at autopsy (*gm.*)	Gain* (*gm.*)	Seromucoid mg. percent tyrosine (*S.E.*)
C Control	0	0	16	42–45	69. 5	1. 4	1. 91 (±0. 080)
D Experimental	0. 25	10	9†	42	74. 8	16. 0	2. 07 (±0. 053)
E "	0. 5	10	10	42	82. 2	20. 3	2. 28 (±0. 047)
F "	2. 5	10	10	42–44	87. 5	23. 7	2. 54 (±0. 128)
G "	10. 0	10	7†	44–45	80. 3	23. 8	2. 74 (±0. 150)
H "	20. 0	10	10	44	84. 0	22. 4	3. 15 (±0. 170)

*Increase in eight during final 10 days of experiment.
†Incompletely hypophysectomized rats were excluded from these groups.

In the group of hypophysectomized rats injected with 2.5 mg. of growth hormone the average increase in weight was 23.7 gm. It was noteworthy that the rats treated with 10.0 and 20.0 mg. of growth hormone did not show a significantly greater response than those treated with 2.5 mg.

The seromucoid level of the hypophysectomized controls was significantly lower, 1.91 mg. percent tyrosine, than that of the intact controls, 2.25 mg. percent tyrosine (tables 1 and 2). In all groups of hypophysectomized rats treated with growth hormone the seromucoid levels were increased and the increase in seromucoid was a function of the logarithm of growth hormone dosage (text-fig. 1). In the hypophysectomized rats injected with a total of 20.0 mg. of pituitary growth hormone the increase in blood seromucoid exceeded that of the intact controls injected with 52.5 mg. of growth hormone.



TEXT-FIGURE 1.—Seromucoid levels as a function of somatotropin dosage in immature female rats.

DISCUSSION

The results of this study differ from those reported by Shetlar *et al.* (*13*). They observed that hypophysectomy in rats lowered total serum polysaccharide, but that the seromucoid was not significantly changed. Also, total serum polysaccharide was elevated after treatment with growth hormone, but the seromucoid remained unchanged. The divergence of the results may be due to differences in experimental conditions, techniques, and the preparations of growth hormone used.

In the present study, the seromucoid levels of hypophysectomized rats were remarkably responsive to growth hormone, whereas those of rats with intact pituitary glands receiving growth hormone changed only slightly. A similar difference in the gravimetric response of hypophysectomized and intact rats has been known for many years. A likely explanation of these differences in response to growth hormone is that

pituitary function of the intact rats is altered by exogenous growth hormone. This is supported by observations of morphologic changes of the anterior pituitary following treatment with somatotropin (*14*).

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A Robertsonian Type of Chromosomal Change in L4946 Mouse Ascites Lymphoma [1, 2]

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SUMMARY

L4946 ascites lymphoma of AKR mice had maintained the karyotype consisting of 41 acrocentric chromosomes for 18 months in our laboratory. Suddenly a large mediocentric chromosome and a heterochromatic minute were formed, apparently at the expense of two acrocentric chromosomes, providing a classic example of a Robertsonian relationship manifesting itself within the neoplastic cell population of a transplantable tumor. The sensitivity to breakage of centromeric heterochromatic regions of the acrocentric chromosomes has been considered as a cause of this type of karyological evolution.—J. Nat. Cancer Inst. 24:1187–1197, 1960.

NEOPLASTIC CELLS of most transplantable tumors in experimental animals are characterized by some karyological features that are not found in normal somatic cells of the species (*1–8*) and that, in succeeding transplant generations, do not persist but may undergo further changes (*5–9*). In fact, the occurrence of new mediocentric chromosomes in many transplantable tumors of the mouse as well as the rat (*2–9*) has been considered one of the most prominent changes.

Recently it was found that L4946 ascites lymphoma of AKR mice maintained in our laboratory underwent karyological changes involving the formation of 1 mediocentric chromosome and 1 heterochromatic minute. The present paper reports results of comparative studies of the karyotypes before and after the changes that suggest a Robertsonian relationship in which 1 mediocentric chromosome is the evolutionary equivalent of 2 acrocentric chromosomes.

MATERIALS AND METHODS

L4946, a transplantable ascites lymphoma of AKR mice originally obtained from the Roscoe B. Jackson Memorial Laboratories, has been

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[2] This study was supported by Public Health Service grant 2597 and the Damon Runyon Memorial Fund grant 428.

maintained in our laboratory since November, 1957, by successive intraperitoneal passages every 6 days in 6- to 8-week-old AKR mice of both sexes from the same laboratories. For transplantation, about 10 million lymphoma cells were used routinely.

The ascites lymphoma cells were examined cytologically 12 times during the 18-month period from November, 1957, to April, 1959—including 7 times during the last 4 months, and 4 times during the 3-month period from June to August, 1959. For the examination, ascites tumor fluid was sampled 2 days after transplantation to obtain a lymphoma-cell population having less frequent abnormal mitoses and fewer wide variations in chromosome numbers. From the sample, Feulgen squash preparations were made according to the method previously described (*10*).

Detailed karyological analyses were made with more than 100 metaphase and 20 to 30 prophase lymphoma cells in each sample. In addition to the lymphoma cells, lymphocytes from lymph nodes and other somatic cells were similarly investigated.

RESULTS

Normal Cells of the Mouse

In the diploid spermatogonial chromosome complement of *Mus musculus*, as previously reported (*10*, *11*), all 19 homologous pairs of autosomes are acrocentric, with one small block of heterochromatin at the proximal end (centromeric heterochromatin) and another smaller block at the distal end (telomeric heterochromatin). Long arms of both the acrocentric X and Y chromosomes are made almost entirely of heterochromatin, strongly demonstrating positive heteropyknosis during meiosis as well as mitosis.

However, the degree of allocycly shown by the X as well as the Y chromosomes in both sexes is reduced to a minimum in certain somatic cells, including the lymphocytes. The heterochromatic sex chromosomes of the lymphocyte appear isopyknotic to the mostly euchromatic autosomes, as described in a previous paper (*12*). Even so, at prophase these sex chromosomes can still be distinguished from the autosomes because, unlike the autosomes, they stain uniformly along their entire length, lacking positive heteropyknotic regions at their proximal and distal ends.

The largest pair of autosomes of *M. musculus* usually measures about 8 μ in length and the smallest about 2 μ. The X at metaphase is about 6 μ; the Y, about one third this size.

L4946 Cells Before April, 1959

All 12 cytologic studies from November, 1957, to April, 1959, showed no changes in karyological characteristics of L4946 in 2-day-old transplants. As an example, findings from the sample obtained March 15, 1959, were as follows: Of 500 metaphase complements casually examined,

431 had 40 ± 5 chromosomes, 45 had 80 ± 5 chromosomes, 9 had 60 ± 5 chromosomes, and the remaining 15 did not belong to any of these categories.

One hundred of the 431 metaphase complements with 40 ± 5 chromosomes were subjected to critical examination. In 92 of them, the karyotype consisted of 41 acrocentric chromosomes (figs. 1 and 2). No mediocentric chromosome or minute segment was found. The largest of the 41 acrocentric chromosomes did not exceed 9 μ in length; the smallest was still about 2 μ.

At prophase, each of these 41 acrocentric chromosomes demonstrated a recognizable degree of positive heteropyknosis at its proximal and distal ends (fig. 3). A centromeric heterochromatin at the proximal end is always more outstanding than a smaller telomeric heterochromatin at the distal end. There were, however, no uniformly stained chromosomes without heteropyknotic ends, similar to sex chromosomes in normal lymphocytes.

L4946 Cells After June, 1959

At the end of June, 1959, a large mediocentric chromosome and one or more minutes appeared in the karyotype of hyperdiploid neoplastic cells. Three analyses made in July and August, 1959, revealed the same characteristics. The findings from the sample obtained on August 14, 1959, will serve as an example: Of 500 metaphase complements casually examined, 428 had 40 ± 5 chromosomes, 51 had 80 ± 5 chromosomes, 14 cells had 60 ± 5 chromosomes, and the remaining 7 did not belong to any of these categories.

One hundred of the 428 metaphase complements with 40 ± 5 chromosomes were examined closely; 96 had 1 large mediocentric chromosome in addition to acrocentric chromosomes. One of the remaining 4 complements had 2 mediocentric chromosomes, while the remaining 3 had none. In 88 of the 100 complements, the karyotype consisted of 39 acrocentric chromosomes, 1 large mediocentric chromosome, and, as a rule, 1 minute. Seventy-three complements had 1 minute, 6 had 2 minutes, and 1 had 3 minutes, whereas 8 had no minutes. The fact that some cells had more than 1 minute and some had none indicates the mitotic instability of the minute (figs. 4 and 5).

Each arm of the large mediocentric chromosome measured about 5 μ in length, whereas the largest and smallest of the 39 acrocentrics still measured about 8 and 2 μ respectively, excluding, of course, the heterochromatic minute.

Prophase figures with 39 acrocentric, 1 large mediocentric, and 1 minute chromosome were examined. The 39 acrocentric chromosomes had small, positively heteropyknotic regions at their proximal and distal ends, similar to those observed in the somatic cells (fig. 6). The large mediocentric chromosome, on the other hand, had a prominent, positively heteropyknotic region at the middle of its length (centromeric hetero-

chromatin). In addition positive heteropyknosis, although less conspicuous, was recognized at both ends (telomeric heterochromatin). The minute chromosome was entirely positively heteropyknotic.

By counting each arm of the mediocentric chromosome as 1, and disregarding the heterochromatic minute, the chromosome number remained 41, as it had been during the previous 18 months.

There was little difference in the pathophysiology of the neoplasm before and after the change in the karyotype. The growth rate as estimated by daily increase in total packed-lymphoma-cell volume, the longevity of animals, and the histology of invaded organs showed only negligible variations (*13*).

DISCUSSION

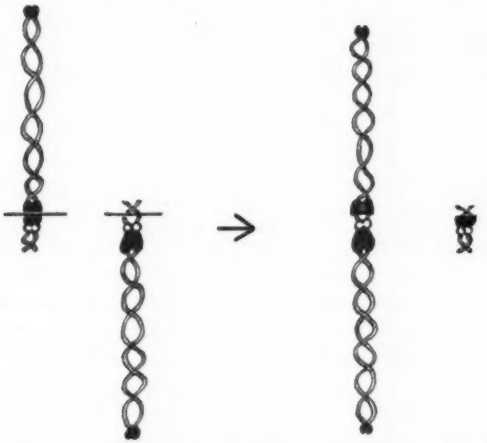
In the transplantable L4946 mouse ascites lymphoma maintained at our laboratory, lymphoma cells with 41 chromosomes were predominant, establishing the stem-cell lineage. All 41 chromosomes were acrocentric, at prophase demonstrating heteropyknotic regions at both the proximal and distal ends. Comparison of these chromosomes with the autosomes of normal lymphocytes leads to the suggestion that all of them are of autosomal origin. The sex chromosomes with the morphology observed in normal lymphocytes were missing.

The stem-cell lineage described continued until April, 1959. In June it was noted that the chromosome complement consisting of 39 acrocentric chromosomes, 1 mediocentric chromosome, and 1 or more minutes became predominant, establishing a new stem-cell lineage. A karyological evolution must have occurred sometime between April and June.

The newly present mediocentric chromosome, which had not been seen before, consisted of 2 arms of about equal length joined by the kinetochore region. There was a small telomeric heterochromatin region at the distal end of each arm. A prominent block of heterochromatin was adjacent to its kinetochore. The accompanying minute was entirely heteropyknotic.

These findings suggest that 2 large acrocentric chromosomes of about equal size were involved in the formation of these chromosomes (text-fig. 1). Very likely, breaks took place at the centromeric regions of 2 acrocentric chromosomes, 1 distal to and the other proximal to their kinetochores. By subsequent fusion of their broken ends, a large mediocentric chromosome was formed with a kinetochore contributed by 1 of the 2 acrocentric chromosomes involved. The kinetochore of the remaining acrocentric chromosome, carrying with it a part of the centromeric heterochromatin and an extremely short second arm, became a minute. Judging from its mitotic instability, a heterochromatic minute may not survive many mitoses.

As to the way in which 2 acrocentrics unite by centric fusion to form a single mediocentric chromosome, three possibilities have been considered by White (*14*).



TEXT-FIGURE 1.—A schematic drawing illustrating the formation of a mediocentric chromosome from 2 acrocentric chromosomes of about equal length. Heteropyknotic regions are *solid black*. Positions of breaks are indicated by *straight lines*.

1. The centric fusions may be produced by direct adhesion of chromosome ends without breaks. The mediocentric chromosome thus formed has 2 kinetochores situated very close together.

2. Two acrocentric chromosomes, after breaks on the extremely short second arms, fuse together at the broken ends, resulting in the formation of a mediocentric chromosome with 2 kinetochores and a minute acentric fragment. The argument against these two possibilities is that such a mediocentric could not possibly pass through mitoses without being broken, as 2 kinetochores on the same chromatid work to opposite poles at anaphase.

3. A mediocentric chromosome with a single kinetochore can be formed only by a break occurring just proximal to the kinetochore in 1 acrocentric chromosome, and just distal to it in the other, followed by a mutual translocation. A minute containing a kinetochore is also formed. This was the exact situation in the present study.

Findings from experiments with various alkylating agents (*13*) indicate that centromeric heterochromatic regions of the acrocentric autosomes of *M. musculus* are exceptionally sensitive to breaks. Further studies on sensitivity to breakage of terminally located centromeric heterochromatin may help interpret the frequent formation of a mediocentric chromosome at the expense of 2 acrocentrics.

Formation of a mediocentric chromosome from 2 acrocentrics was emphasized by Robertson, in 1916, as an important source of variation in the number and shape of chromosomes within the species. Recent studies by Wahrman on the mantids genus *Ameles* provided a significant example of a Robertsonian relationship manifesting itself within as well as between the closely related species. The populations of 3 species of

the mantids genus *Ameles* are heterogeneous with regard to the chromosome complements of their members.

In each species this structural polymorphism depends on 1 or several chromosomes which may be represented either as 1 metacentric or 2 acrocentric elements. The diploid chromosome numbers vary from 19 to 29, but the total number of arms remains constant, as does the mean amount of DNA (deoxyribonucleic acid) per nucleus (*15*, *16*). In mammals, well-documented instances of chromosomal phylogeny conforming to Robertson's hypothesis have been reported in the rodent genus *Gerbillus* (*17*). The diploid chromosome numbers of 3 species of Old World monkeys vary from 42 to 60, yet the mean diploid DNA values were found to be essentially the same (*18*).

It appears that structural rearrangements of the chromosomes, with little or no change in the total amount of material involved, play a more important role than polyploidy in the evolution of living organisms belonging to the animal kingdom. In organisms capable of sexual reproduction, formation of mediocentrics from acrocentrics results in differences in the number of linkage groups, the recombination indices, and the relative positions of certain genes. Structural rearrangements of this type may determine differences in adaptive values of their carriers.

In neoplastic cells, too, changes in the relative positions of certain genes caused by linkage of 2 chromosomes may result in similar differences. Autonomously growing neoplastic cells of a well-established transplantable tumor may be regarded as a strain of organisms characterized by a more or less fixed karyotype. The karyotype of neoplastic cells often undergoes structural rearrangements producing sublines of the tumor (*5*, *19*).

The particular strain of L4946 that has been described changed its karyotype from 41 acrocentric chromosomes to the complement of 39 acrocentrics, 1 large mediocentric, and 1 heterochromatic minute chromosome. The large mediocentric chromosome was apparently formed at the expense of 2 acrocentrics. Since little or no euchromatin is involved in a minute, the loss of one would not change the total amount of genetically important euchromatin. Nevertheless the possibility of a change in the relative positions of certain genes cannot be overlooked. Although the present study of L4946 failed to show any changes in the growth pattern and other criteria considered, it cannot be concluded on this basis alone that genetic alterations were minor, despite the marked changes in the chromosome complements of the stem cells.

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PLATES

PLATE 116

Photomicrographs of hyperdiploid complements of L4946 ascites lymphoma sampled on March 15, 1959. A Leitz Panphoto with 90 × 100 lenses was used.

FIGURES 1 AND 2.—At metaphase. *Note* 41 acrocentric chromosomes.

FIGURE 3.—At prophase. Schematic sketch (3b) details the 41 acrocentric chromosomes, each with heteropyknotic regions at the proximal and distal ends. No uniformly stained chromosomes, similar to sex chromosomes, are seen.

1

2

3a

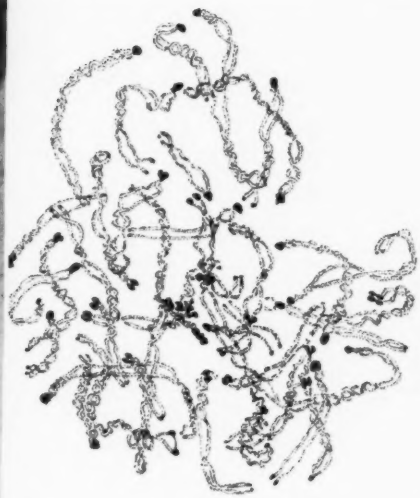
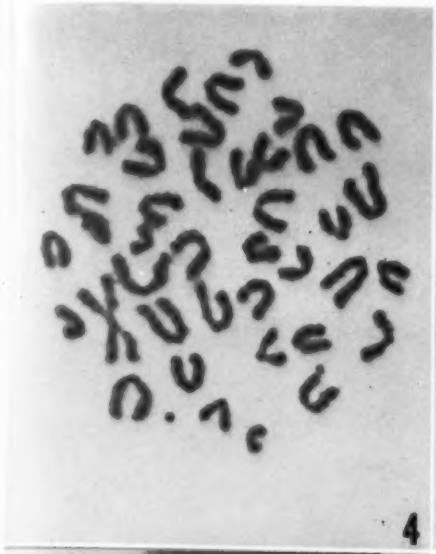

3b

PLATE 117

Photomicrographs of hyperdiploid complements of L4946 ascites lymphoma sampled on August 14, 1959. A Leitz Panphoto with 90 × 100 lenses was used.

FIGURES 4 AND 5.—At metaphase. *Note* 39 acrocentric chromosomes, 1 large mediocentric chromosome, and 1 minute.

FIGURE 6.—At prophase. In schematic sketch (6b), *note* 39 acrocentric chromosomes, each with heteropyknotic regions at the proximal and distal ends; 1 *arrow* points to a large mediocentric chromosome with centromeric and telomeric heterochromatin, and the other *arrow* to a heteropyknotic minute.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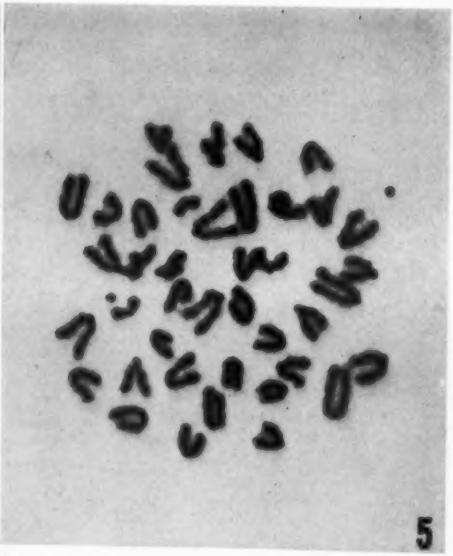
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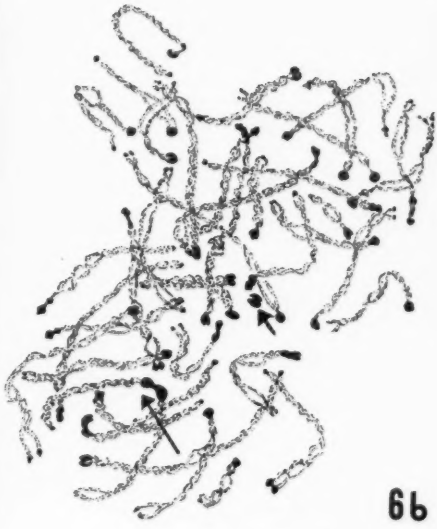
6a


6b

Postcoupling, Noncoupling, and Fluorescence Techniques for the Demonstration of Alkaline Phosphatase [1]

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SUMMARY

The development and use of new postcoupling, noncoupling, and fluorescence techniques for the demonstration of alkaline phosphatase are described. The techniques utilize new substrates, derivatives of hydroxynaphthoic acid, and complex amines. Three of the most useful substrates are 5,6,7,8-β-tetralol carboxylic acid-β-naphthylamide phosphate (I), 2-hydroxy-3-naphthoic acid-2-aminoanthraquinonylamide phosphate (II), and 2-hydroxy-3-naphthoic acid-aminoazobenzanilide phosphate (III). The techniques, which are designed primarily for use with paraffin-embedded tissues, give sharp localizations. These compare with the best results obtained by the simultaneous-coupling technique. In the case of one substrate, an anthraquinone derivative (II), postfixed frozen sections give useful results. The naphthol released from substrate I is a fluorochrome and is the basis of a new avenue of approach in enzyme histochemistry, namely, the visualization of enzyme activity by fluorescence. This and another substrate also liberate naphthols, which are visible under optical magnification without postcoupling. This application is referred to as a *noncoupling* procedure. For most purposes, a postcoupling procedure utilizing substrates II or III is recommended. The incubating solutions are stable, and subsequent postcoupling may be accomplished with a large variety of diazonium salts. These techniques also lend themselves to the demonstration of hydrolytic enzymes other than alkaline phosphatase.—J. Nat. Cancer Inst. 24: 1199–1217, 1960.

POSTCOUPLING TECHNIQUES are probably ideal for the demonstration of alkaline phosphatase as well as other enzymes in that any possible inhibitory effect of diazonium salt is eliminated. In addition, an elevated temperature may be maintained during the incubation period. Techniques using naphtholic substrates, which liberate an opaque deposit or colored dye, would also fall into the same category, but could be referred to as *noncoupling* procedure.

The application of fluorescence techniques to enzyme histochemistry would also represent an advance in that the visualization of a fluorochrome would permit detection of very low levels of activity.

In several previous reports the author discussed the use of several new

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[2] National Institutes of Health, U.S. Department of Health, Education, and Welfare.

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phosphatase substrates as postcoupling reagents in the study of bone and tooth structure (*1*, *2*). The present report will deal with the use of these new reagents as histochemical reagents for the demonstration of alkaline phosphatase in normal, soft, and hard tissues, as well as in neoplasms.

The product of hydrolysis, a complex naphthol, may be visualized by postcoupling with a diazonium salt, by its ability to fluoresce under ultraviolet light, or by its optically refractile character.

METHODS

Substrates

All substrates were derived from complex naphthols obtained from 2-hydroxy-3-naphthoic acid (BON) and certain aryl amines. Amides from BON and the following amines were used:[3]

(1) β-amino anthraquinone;
(2) *p*-aminoazobenzene;
(3) *p*-aminobenzophenone;
(4) 2-nitro-3-aminodibenzofuran;
(5) dehydrothio-*p*-toluidine.

In addition, a unique naphthol, 5,6,7,8-,β-tetralol carboxylic acid-β-naphthylamide, was used (*3*).

The aforementioned naphthols were prepared by standard techniques involving the condensation of BON with amine in the presence of phosphorus trichloride (*4–6*). Equivalent quantities of amine and 2-hydroxy-3-naphthoic acid and approximately 400 ml. xylene were placed in a three-neck flask. The contents were heated to 80° C. (with stirring) and 8.0 cc. phosphorus trichloride (PCl_3) was added. The mixture was then refluxed for several hours and the resulting mixture was decanted, filtered, and washed with xylene. The naphthol was purified by recrystallization from nitrobenzene or a mixture of *N*,*N*-dimethylformamide(DMF)-methanol.

A similar procedure involved solution or suspension of equimolar quantities of amine and BON in pyridine, which was then heated to 70° C. (*4*). Phosphorus trichloride was then added over a period of 30 minutes, and this mixture was heated for several hours.

Preliminary studies indicate that carbodiimides may also be useful condensing agents to form the anilides under very mild conditions (*1*). For example, amine, BON, and a carbodiimide (*e.g.*, *N*,*N'*-di-*p*-tolylcarbodiimide or *N*,*N'*-dicyclohexylcarbodiimide) are suspended or dissolved in an inert solvent (tetrahydrofuran) and allowed to stand overnight. The urea is removed by filtration and the excess solvent evaporated.

The histochemical substrates were obtained by phosphorylating the aforementioned naphthols with phosphorus oxychloride, according to a previously described technique (*6*, *7*). The appropriate naphthol, suspended in tetrahydrofuran, is treated with phosphorus oxychloride.

[3] Preliminary test samples of some naphthols were obtained from E. I. du Pont and Co., Wilmington, Del., and the Pfister Chemical Works, Ridgefield, N.J.

Present in the mixture may be ethylenediaminetetraacetic acid (EDTA) (50.0 mg.), *p*-toluenesulfonic acid monohydrate (50.0 mg.), and sodium chloride (500.0 mg.), which act as catalysts. (When phosphorylation with phosphorus oxychloride is employed, impurities in the form of secondary and other phosphate esters may be present. These, however, do not adversely affect the histochemical reaction. If a purer substrate is desired, further purification or the use of other synthetic routes may be employed.) Several readily available commercial naphthols were also phosphorylated. These were:

(1) naphthol AS-S, 3-hydroxy-*N*-(2-methoxy-3-dibenzofuryl)-2-naphthamide;

(2) naphthol AS-BI, 6-bromo-3-hydroxy-2-naphth-*o*-anisidide.

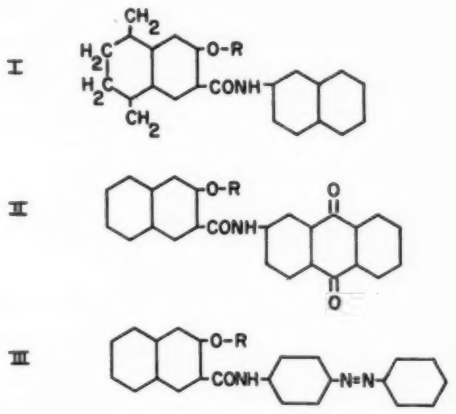
These two, however, tended to yield more diffusion artifacts than the other substrates and thus were used only in preliminary studies.

The substrates that were most often used were (text-fig. 1):

I. 5,6,7,8-β-tetralol carboxylic acid-β-naphthylamide phosphate;

II. 2-hydroxy-3-naphthoic acid-2-aminoanthraquinonylamide phosphate;

III. 2-hydroxy-3-naphthoic acid-aminoazobenzanilide phosphate.



R= Phosphate grouping

The substrates were used as follows:

(1) Approximately 5 mg. substrate (excess) was placed in a 50 ml. Erlenmeyer flask and 0.25 ml. *N*,*N*-dimethylformamide (DMF) [4] added. In the case of I, 0.5 ml. DMF was used. The flask was then gently agitated in order to dissolve the substrate.

(2) Twenty-five ml. distilled water was then added, followed by 25.0 ml. 0.2 M tris buffer, *p*H 8.3 to 9.2. In general *p*H 8.7

[4] Matheson, Coleman, and Bell, Norwood, Ohio.

tris buffer was used [24.2 gm. tris(hydroxymethyl)aminomethane, 16.5 ml. N HCl, and distilled water to make 1 liter].

(3) Two drops of 10 percent $MgCl_2$ were added and the solution was shaken several times and filtered through folded filter paper into a Coplin jar. The resulting solution is opalescent to clear in appearance.

(4) Incubations may be carried out at room or elevated temperature (*e.g.*, 37° C.). Frequently, the Coplin jar was placed in a 60° C. paraffin oven for 15 minutes in order to warm the solution rapidly and then maintained at room temperature for the remainder of the incubation period. Total incubation periods ranged from 60 to 180 minutes.

(5) Following incubation, the slides were washed in running tap water for several minutes and then transferred (except in the case of the noncoupling and fluorescence techniques) to a suitable diazonium salt solution.

(6) The diazo coupling solution consisted of approximately 50 mg. diazonium salt, 20.0 ml. 0.2 M sodium acetate, and 30.0 ml. distilled water. This mixture was shaken and filtered into a Coplin jar. (In some cases addition of 1.0 ml. acetone to the Coplin jar will facilitate coupling.) The slides were placed in the diazo solution and allowed to stand for 10 minutes at room temperature. They were then washed in running water and mounted in glycerol-gelatin (without phenol), 25 percent polyvinyl acetate in 80 percent ethanol (*8*), or polyvinyl pyrrolidone (*9*).

The following stable diazonium salts were used:

(1) Fast Garnet GBC salt,[5] diazotized *o*-aminoazotoluene. This gave dark-reddish shades and is recommended for preliminary trials.

(2) Fast Red RL salt,[6] diazotized 5-nitro-2-aminotoluene. This gave reddish shades.

(3) Fast Red B salt,[6] diazotized 5-nitro-2-amino anisole. This also gave reddish shades.

(4) Fast Red Violet LB salt,[5] diazotized 5-benzamide-4-chloro-2-toluidine. This gave red and red-brown shades.

(5) Fast Navy Blue salt RA,[5] diazotized toluidine. This gave blue shades.

(6) Fast Blue BBN salt,[6] diazotized 5-amino-2-benzoylamino-1,4-diethoxybenzene. This gave dark-blue shades.

(7) Fast Black salt G,[5] diazotized *p*-aminodiphenylamine-azotoluene. This gave shades of red with substrate II and blackish shades with the other compounds.

A large number of hitherto unused diazonium salts may be employed,

[5] Verona Dyestuffs, Union, N.J.
[6] General Dyestuff Corp., New York, N.Y.

since their enzyme-inhibitory characteristics are of no consequence in postcoupling procedures.

For fluorescence studies, sections incubated in substrate I were washed briefly in 2 changes of 50 percent ethanol and running water and subsequently mounted in glycerol.

Tissues

The aforementioned techniques are designed primarily for use with paraffin-embedded tissues. Either frozen-dried (single or double-embedded) or cold acetone-fixed paraffin-embedded tissues may be used. In some instances fresh-frozen sections mounted on glass slides (Adamstone-Taylor cold-knife technique) were briefly fixed and incubated in substrate II. Fixation was accomplished by immersion in cold neutral formalin for 15 minutes or by brief immersion (1 minute) in acetone, 95 percent acetone-water, and 80 percent acetone-water at room temperature.

The tissues used included rat kidney, liver, spleen, pancreas, and bone (undecalcified 4-day-old hamster and mouse heads, and knee joints). The human neoplasms studied included an adenocarcinoma of the lung, carcinoma of the colon, carcinoma of the liver, and carcinoma of the ovary. The same tumors were used in a previous study, in which AS-phosphates in a simultaneous-coupling method (*7*) were employed. In addition, a human and a mouse osteogenic sarcoma and a human meningioma were studied. Human blood smears on Mylar film (*10*) were also incubated with substrates II and III.

Specificity studies

Representative slides were incubated in substrate solutions to which had been added the following potential inhibitors:

(a) arsenate (sodium arsanilate), 0.01 M;
(b) cyanide (KCN), 0.001 M;
(c) molybdate $(NH_4)_6\ Mo_7O_{24}$, 0.01 M;
(d) cysteine, 0.01 M.

Sections were also immersed in hot water, 90° C., for 10 minutes prior to incubation. Slides were also immersed in a solution of each naphthol for several hours to ascertain if any nonspecific adsorption took place.

OBSERVATIONS

Tissue Staining

Good localizations were obtained with most of the substrates. Substrates II and III yielded extremely sharp staining reactions.

Substrates I and III were unique in that the enzymatically released naphthol could be visualized without subsequent coupling (figs. 1 and 2). This type of technique will be referred to as a *noncoupling* procedure. The naphthol liberated from substrate I was deposited as fine crystals, which sometimes assumed a rather coarse appearance under high magni-

fication. Therefore, it is generally recommended that this substrate be studied under a magnification of 200 × or less. The released naphthol gave a blue-white fluorescence under ultraviolet light and thus constituted the basis of a new enzyme technique (figs. 3 through 8). Substrate III liberated a very homogeneous greenish naphthol which gave highly discrete localizations. Suitable filters may be used to accentuate the contrast. The remaining substrates liberated naphthols which, after coupling, formed homogeneous-appearing dyestuffs, even at the highest magnifications.

All substrates were hydrolyzed more slowly than simple naphthyl phosphates. This, however, did not offer any difficulty since, with the postcoupling procedure, the substrate solution remains clear for hours.

The distribution of stain in the various tissues used was essentially similar to that observed with simultaneous-coupling procedures employing naphthol AS-phosphates (7). Raw frozen sections were unsatisfactory with substrates I and III because a precipitate of coarse dye crystals resulted. However, substrate II gave usable results with raw frozen sections and good localizations when the frozen sections were briefly postfixed in ethanol (fig. 9). Nuclear staining was never seen with any substrate under any condition of incubation.

In rat kidneys a characteristic reaction of the tubular brush borders was observed (fig. 13). The intestine exhibited sharp localizations to the borders of villi (fig. 14). Capillaries were also stained. The adventitia of arterioles exhibited a characteristic reaction that has been observed previously with the simultaneous-coupling procedure (7). There was considerable activity in the basement membranes of skin and oral mucosa (fig. 2). Osteoblasts and osteocytes associated with intramembranous bone were highly active (fig. 10), but no activity was seen in the matrix. Tooth structure showed the same localizations as seen with simultaneous-coupling methods (*1*, *2*). The stellate reticulum and stratum intermedium were highly active, as were the odontoblastic zone and subjacent pulp (fig. 15). The active perichondrium and zone of erosion of calcifying cartilage also stained. Leukocytes in tissue sections, as well as those in human blood smears on Mylar films, were distinctly stained (fig. 11). Certain granulocytes in sections of hamster and bone marrow were also active.

The neoplasms had a staining pattern identical to that observed with the AS-phosphate simultaneous-coupling technique (7) (figs. 12 and 17). The cytoplasmic reaction of the human meningioma was distinct and discrete (figs. 16 and 18).

Inhibitor and Coupling Studies

Heat treatment completely inactivated the enzyme. All of the potential inhibitors had an appreciable effect upon the reaction. Molybdate and cysteine produced complete or almost complete inhibition, while

fluoride reduced the final staining intensity only slightly to moderately. Cyanide and arsanilate had a moderate inhibitory effect.

Most diazonium coupling components gave useful results with the various substrates. Of particular value were Black salt G, Fast Red salt RL, Red Violet LB salt, and Fast Blue salt BBN.

Slides incubated in saturated "naphthol" solutions alone for several hours and then immersed in diazonium solution showed no staining. This indicates that tissue components cannot nonspecifically adsorb and concentrate naphthol from solution in an amount sufficient to produce a visible stain.

DISCUSSION

Postcoupling techniques appear to be an ideal type of histochemical procedure. Extraneous and potentially inhibitory substances such as diazonium salts are eliminated from the incubating solution. In addition, the stability of the solution is enhanced. In the past, postcoupling methods were generally considered unsatisfactory (*11*) because the product of hydrolysis (*e.g.*, naphthol) was diffusable.

The prerequisite of a good postcoupling method is a naphthol or similar compound with very high "substantivity." This term applies to the affinity of dyes or compounds for tissues, and has been discussed in the histochemical as well as dyestuff literature (*12*, *13*). Substantivity is related in part to molecular size, in that increased molecular size may enhance precision of localization. In addition, certain specific features of the molecule *e.g.*, peptide bonds may also confer substantivity. For example, naphthol AS dyestuffs are known to have high substantivity. This is also true of anthraquinone derivatives. Thus, by combining a naphthol and an aminoanthraquinone, a compound of unique properties results. This naphthol, of course, is the basis of substrate II.

Azo-benzene compounds also have substantive qualities, which is apparent from their ability to stain the skin. Substrate III incorporates aminoazobenzene and a naphthol. Thus, in practice, the precision of staining obtained with these new postcoupling substrates is equal to that obtained by the simultaneous-coupling method with AS-phosphates.

The new substrates are also advantageous in that the naphthol is not nonspecifically and constantly adsorbed should any be present in solution (*e.g.*, as a free-naphthol contaminant). This contrasts sharply with results obtained with 6-benzoyl-2-naphthol, which may be "concentrated" from solution by certain tissues and thus give spurious staining (*14*).

As previously mentioned, these techniques are designed for use with the following paraffin-embedded tissues: (1) cold acetone-fixed paraffin-embedded, Gomori technique (*11*); (2) frozen-dried single and double-embedded tissues; (3) freeze-substituted tissues. When raw frozen or even partially fixed frozen tissues are used, the naphthol or final dye may crystallize badly, due to the effect of free lipide, a frequent occurrence

with substrates I and III. This difficulty is not encountered with paraffin-embedded sections. However, in the case of substrate II, useful results are obtained following brief fixation of frozen sections mounted on glass slides (Adamstone-Taylor cold-knife method). Greater cognizance should be taken in histochemistry of the relation between type of fixation and specific technique to be employed. Although a method may give excellent results with paraffin-embedded tissues, the same technique may not be equally suited for frozen sections. It must also be remembered that the enzyme diffusion encountered in poorly fixed or unfixed tissue cannot be compensated by the best of substrates.

The introduction of a histochemical fluorescence technique for the demonstration of alkaline phosphatase represents an innovation in enzyme histochemistry. Of interest is the fact that an enzymatically released naphthol (I) is a fluorochrome. The procedure may be referred to as naphtholic *noncoupling fluorescence technique*. The use of a fluorochrome should markedly increase the sensitivity of phosphatase techniques and other hydrolase techniques as well.

The naphthol released by substrate I, as well as that released by substrate III, is visible under routine optical magnification. This type of method, as previously indicated, may be referred to as *noncoupling*. The application of the new *postcoupling*, *noncoupling*, and *fluorescence methods* to other hydrolytic enzyme systems is now in progress.

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PLATES

All tissues frozen and dried unless otherwise indicated.

PLATE 118

FIGURE 1.—Incisor tooth and adjacent bone of 3-day-old hamster. Noncoupling technique, substrate I. A—bone, B—stratum intermedium, C—odontoblasts. × 143

FIGURE 2.—Basement membrane of oral epithelium from 2-day-old hamster. Noncoupling technique, substrate I. × 143

FIGURE 3.—Rat kidney. Fluorescence technique, substrate I. × 100

FIGURE 4.—Human gingiva. Fluorescence technique, substrate I. *Note* reaction of capillaries. × 80

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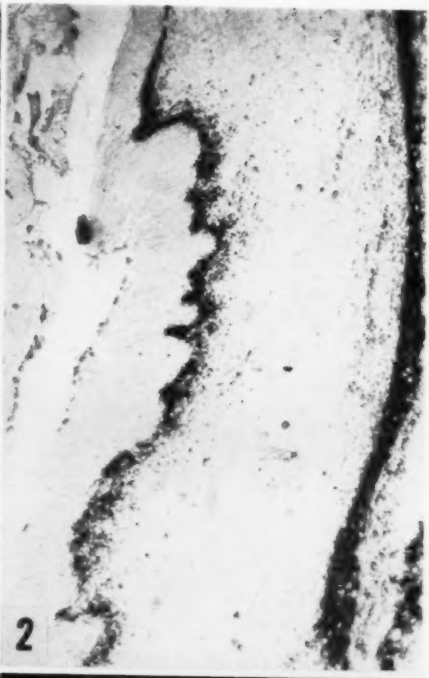
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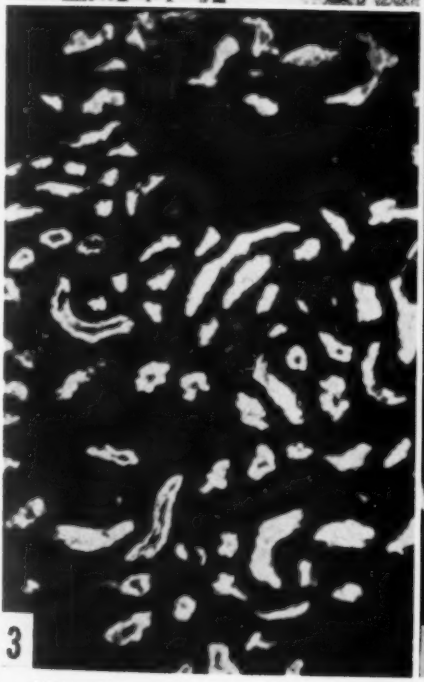
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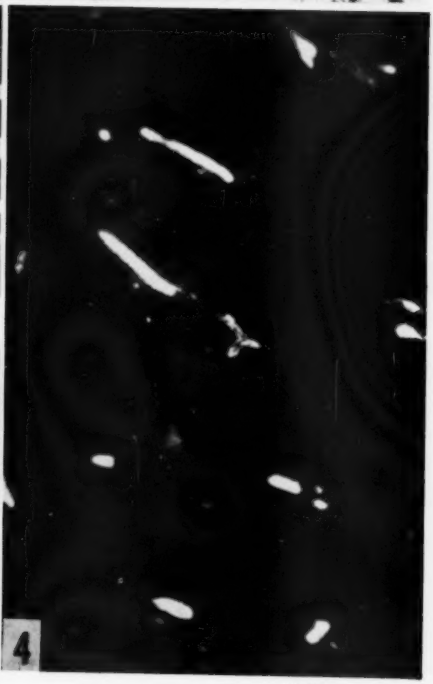
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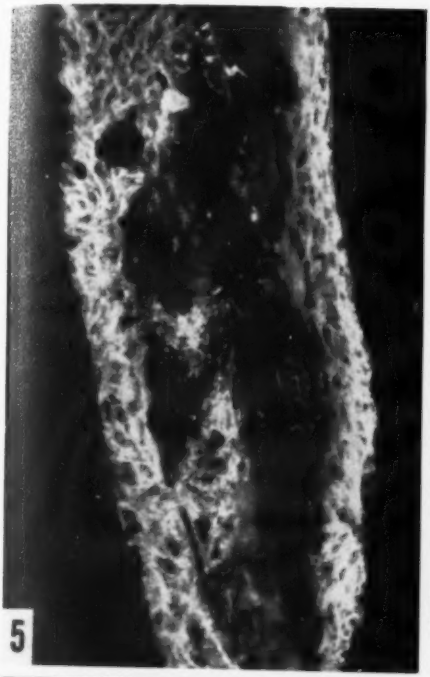
PLATE 119

FIGURE 5.—Intramembranous bone of palate (3-day-old hamster). Fluorescence technique, substrate I. *Note* intense reaction of osteoblasts. × 100

FIGURE 6.—Higher magnification of area similar to that described in figure 5. Fluorescence method, subtrate I. Cytoplasm of osteoblasts reacts strongly. × 500

FIGURE 7.—Molar tooth of 3-day-old hamster. Fluorescence method, substrate I. A—stratum intermedium, B—ameloblasts, C—odontoblasts (enamel and dentin matrices are autofluorescent), D—pulp. × 80

FIGURE 8.—Rat duodenum. Fluorescence method, subtrate I. × 100



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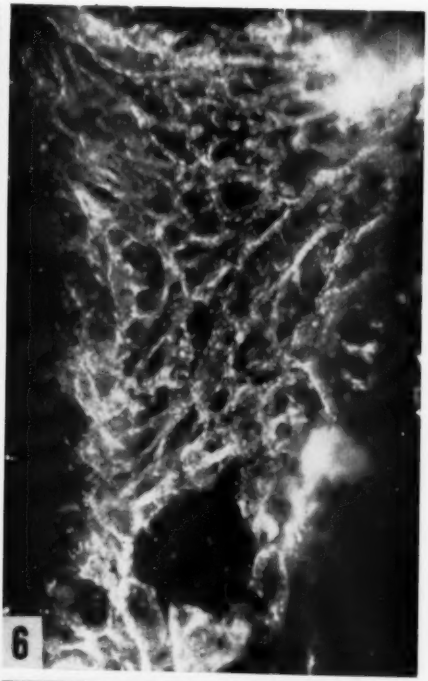

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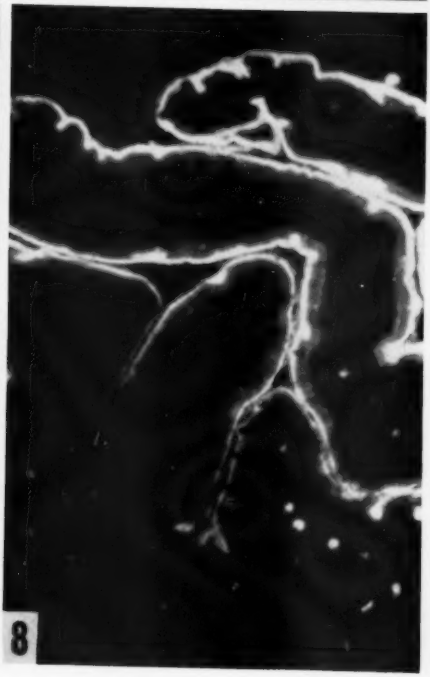

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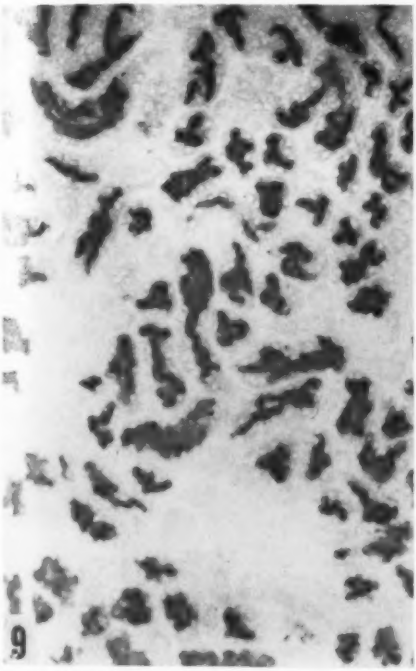
PLATE 120

FIGURE 9.—Raw-frozen rat kidney postfixed in ethanol, subtrate II, postcoupled with Red Violet LB salt. × 143

FIGURE 10.—Intramembranous bone formation in 2-day-old hamster head, subtrate II. *Note* intense reaction of osteoblasts. × 285

FIGURE 11.—Rat liver, subtrate II, postcoupled with Black G salt. *Note* staining of bile canaliculi and reaction of leukocytes. × 285

FIGURE 12.—Carcinoma of lung, subtrate II, postcoupled with Red RL salt. × 143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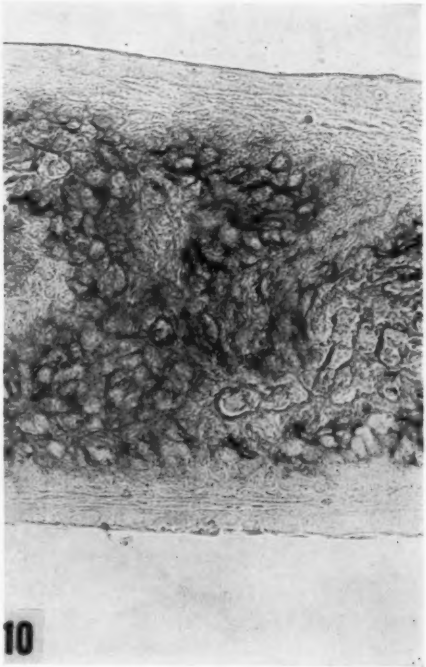
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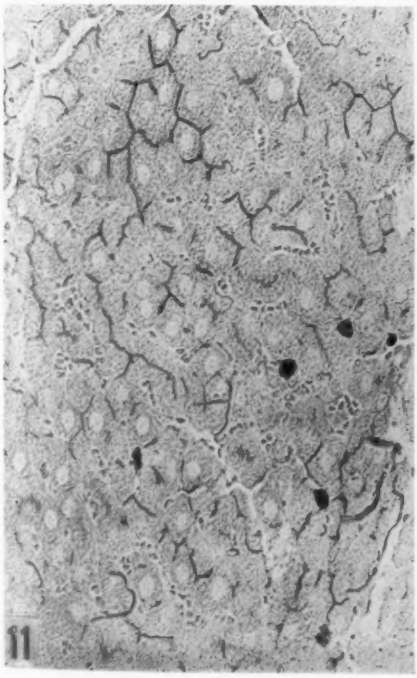
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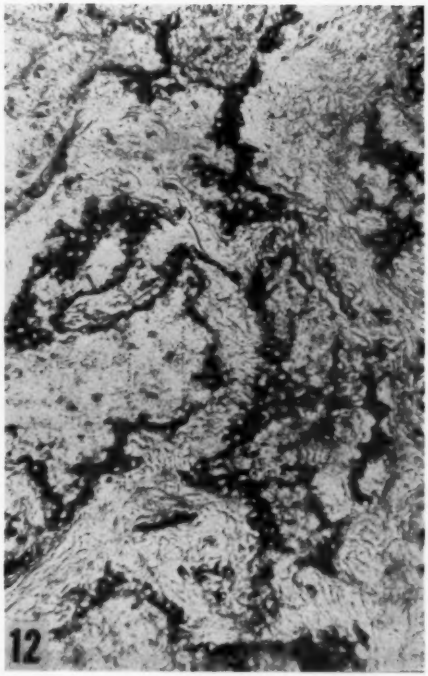
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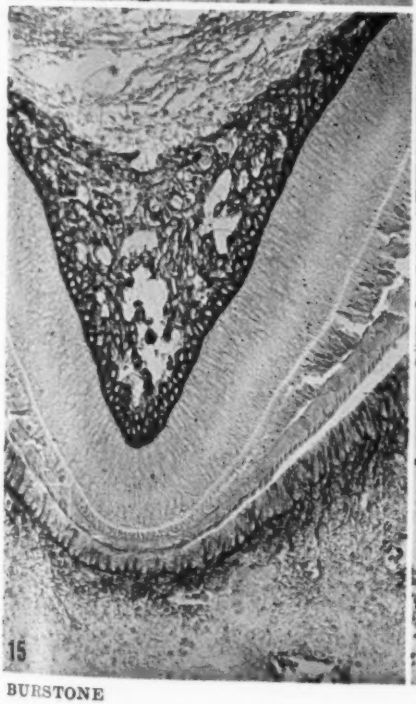
PLATE 121

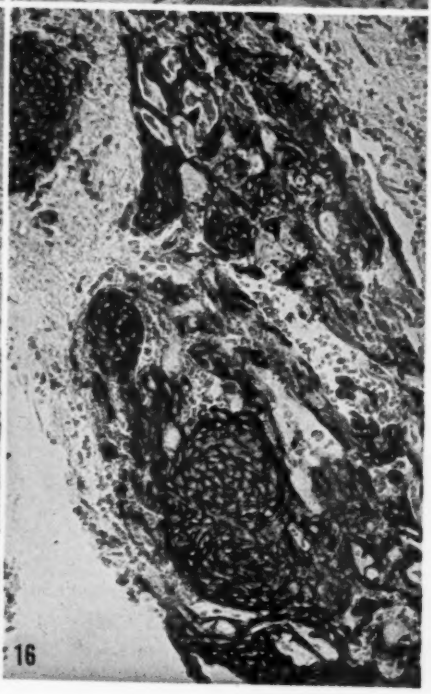
FIGURE 13.—Rat kidney, subtrate III, postcoupled with Blue BBN salt. Lithium carmine counterstain. × 143

FIGURE 14.—Rat intestine, substrate III, postcoupled with Red RL salt. Hematoxylin counterstain. × 117

FIGURE 15.—Molar tooth of 3-day-old hamster, subtrate II, postcoupled with Black G salt. Lithium carmine counterstain. × 143

FIGURE 16.—Human meningioma, subtrate II, postcoupled with Black G salt × 143





BURSTONE

PLATE 122

FIGURE 17.—Mouse osteogenic sarcoma. Fluorescence technique with substrate I. × 50

FIGURE 18.—Lower-power magnification of figure 17. Fluorescence method with substrate I. × 50

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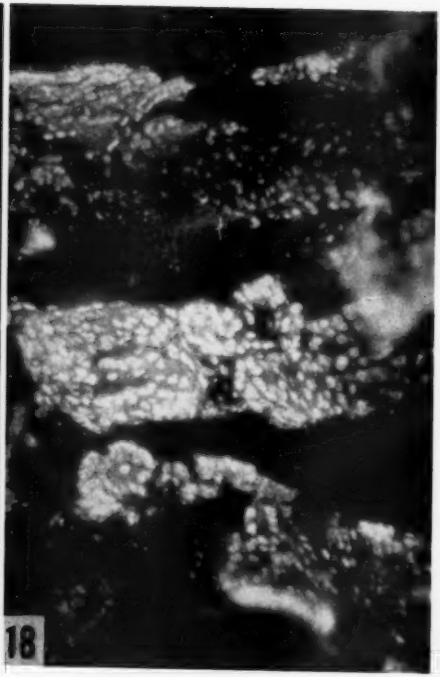
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The Growth of Estrogen-Induced Interstitial-Cell Testicular Tumors in BALB/c Mice [1]

HOWARD B. ANDERVONT, MICHAEL B. SHIMKIN, *and* HARRY Y. CANTER,[2] *Laboratory of Biology and Biometry and Epidemiology Branch, National Cancer Institute,*[3] *Bethesda, Maryland*

SUMMARY

Strain BALB/c male mice, 2 months old, were implanted with stilbestrol-cholesterol pellets, which were removed either when one or both testes became enlarged to 9 to 10 mm. in length, or 2 weeks later. The interstitial-cell tumors thus induced were measured weekly and the growth patterns analyzed. There were at least 7 separable growth patterns, which again could be combined into tumors that grew, persisted without continued growth, or regressed; a proportion of the latter recurred after variable periods. Bilateral tumors behaved as variably as unilateral tumors. Mice exposed to estrogen 2 weeks longer after the appearance of a tumor had more tumors that grew progressively and fewer that regressed than mice whose pellets were removed as soon as they developed tumors. Exposure of mice to testosterone or to progesterone did not affect the rate of recurrence of tumors that had regressed. The variability of growth patterns indicates no single evolutionary progression for the tumors but a great number of general and local factors in what biologically must be an unstable system between tumor and host.—J. Nat. Cancer Inst. 24: 1219–1237, 1960.

THE BIOLOGIC and histologic properties of testicular tumors of interstitial-cell origin, which occur in BALB/c mice implanted subcutaneously with stilbestrol-cholesterol pellets, have been reported in several publications from this laboratory (*1–3*). A finding of recent interest was that the removal of estrogen pellets from mice that had developed definite gross tumors of the testes resulted in the continued growth of some tumors, while others regressed so that the testes returned to normal size. Some of these regressed tumors recurred spontaneously, and prompt recurrence could be achieved in others by the reimplantation of another estrogen pellet.

This report deals with further studies on the estrogen-induced testicular tumors and their course after removal of the hormonal stimulus. Particular attention was paid to the standardization of conditions and definitions in order that the growth patterns of a large number of testicu-

[1] Received for publication November 24, 1959.
[2] We are indebted to Miss Joan M. Gurian and Mr. Peter M. Shimkin for assistance in the analysis of these data.
[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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lar tumors could be followed. The great variability of the life history of these tumors is demonstrated; this variability indicates that there is no simple pattern of evaluation for these tumors and that a great number of general and local factors must influence the progression, regression, and the fluctuations of what biologically must be an unstable system.

The data showed that, in mice exposed to only 2 weeks of estrogenic stimulation after the appearance of definite tumors, more tumors grew progressively and fewer regressed than in mice whose estrogen pellets were removed as soon as they developed definite tumors. Also, the exposure of mice to testosterone or to progesterone did not affect the rate of recurrence of regressed tumors.

MATERIALS AND METHODS

Two separate experiments are reported. In experiment 317, male mice of the BALB/c strain received, subcutaneously in the right axilla, pellets weighing approximately 5 mg. and containing 20 percent stilbestrol in cholesterol. All mice were 2 months old at the time of implantation of estrogen.

The mice were maintained in plastic boxes, with an unlimited supply of Purina laboratory chow and water. They were examined every week, and when a testis became enlarged, its long axis was measured and recorded weekly.

When one or both of the testes reached 9 to 10 mm. in length, which was taken as the criterion for the presence of a gross tumor, the animal was set aside and 14 days later the stilbestrol pellet was excised. Each testis continued to be measured weekly until the size of the mass and/or the general condition of the animal indicated early fatal termination and possible loss of the autopsy. The measurements of the testes were plotted on graph paper for each animal, and the patterns analyzed.

Mice in which testicular tumors had regressed for over 2 months after the removal of the stilbestrol pellets were divided into 2 groups and paired controls: One group was implanted subcutaneously with pellets of solid testosterone weighing 5 mg., the second group was implanted with pellets of solid progesterone weighing 15 mg., and the controls were not treated.

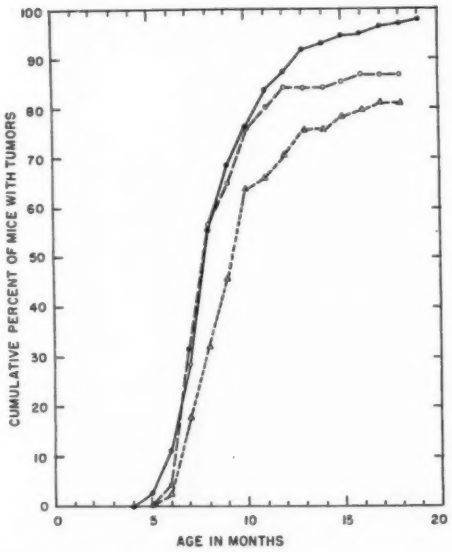
All mice were autopsied unless excessively autolyzed, and sections of the testes and gross abnormalities were obtained for histologic examination. The tissues were fixed in Tellyesnicky's fluid (2 parts 40% formaldehyde solution, 20 parts 70% alcohol, and 1 part acetic acid), blocked in paraffin, cut, and stained with hematoxylin and eosin.

Experiments 300 and 300A.—The conditions were the same as those of the previous experiment, except that the pellets were excised as soon as one or both of the testes reached 9 to 10 mm. in the longest axis. Approximately half the animals received pellets containing 20 percent stil-

bestrol (expt. 300A) and the remainder were implanted with pellets containing 10 percent stilbestrol (expt. 300).

EXPERIMENTAL PROCEDURES AND RESULTS

A total of 589 mice received stilbestrol pellets. Tables 1 to 3 summarize the results for the 3 groups of animals, and text-figure 1 graphs the cumulative frequency of the tumors with the actuarial method of making allowance for animals dying without tumors.



TEXT-FIGURE 1.—Cumulative appearance of testicular tumors in strain BALB/c mice implanted subcutaneously with stilbestrol-cholesterol pellets. *Solid line* and *solid circles*, 20 percent stilbestrol pellets, removed 2 weeks after enlargement of testis to 9 to 10 mm. in length (expt. 317). *Dash line* and *open circles*, 20 percent stilbestrol pellets, removed when testis enlarged to 9 to 10 mm. (expt. 300A). *Dash line* and *open triangles*, 10 percent stilbestrol pellets, removed when testis became enlarged to 9 to 10 mm. (expt. 300).

It is important to emphasize that in all experiments the time recorded for the occurrence of a testicular tumor was when the testis measured at least 9 mm. in length, as compared with the normal length of 6 to 7 mm. This criterion was selected after experience had shown that the term "enlarged" testis was unsatisfactory because such enlargement could persist for a time, or regress, in mice that continued to carry estrogen pellets. In pellet-carrying mice, small, yellow areas were visible in testes of normal size; these areas, on examination with the microscope, consisted of foci of interstitial-cell hyperplasia. When the testis was enlarged to 9 mm., usually all but the cortical rim was composed of an interstitial-cell mass.

TABLE 1.—Experiment 317. Occurrence of interstitial-cell tumors of testes in BALB/c mice implanted subcutaneously with 20 percent stilbestrol-cholesterol pellets at 2 months of age—pellets removed *2 weeks after* testes reached 9 to 10 mm. in length

Mice	Age in months: 2	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Total	Mean age (*months*)
Total	394	359	357	352	347	343	339	337	334	333	332	331	331	329	328	326	323		
Dying without tumor		35	2	5	5	4	4	2	3	1	1	1		2	1	2	3	71	7. 3
Tumor in right testis			3	16	30	43	10	12	11	3	6	4	1	0	1	1		141	8. 6
Tumor in left testis			7	13	23	29	15	10	6	4	6		2	1			1	117	8. 5
Simultaneous bilateral tumors					8	21	18	4	6	4	1		1		2			65	9. 3
Total developing tumors			10	29	61	93	43	26	23	11	13	4	4	1	3	1	1	323	8. 7

TABLE 2.—Experiment 300A. Occurrence of interstitial-cell tumors of testes in BALB/c mice implanted with 20 percent stilbestrol-cholesterol pellets at 2 months of age—pellets removed as soon as testes reached 9 to 10 mm. in length

Mice	Age in months: 2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Total	Mean age (*months*)
Total	101	95	91	86	85	85	83	83	83	83	83	83	83	79	73		
Dying without tumor		6	4	5	1		2							4	6	28	10. 5
Tumor in right testis			1	17	10	3	5	3	2			1				42	8. 4
Tumor in left testis			3	4	5	4	3	1	1				1			22	8. 7
Simultaneous bilateral tumors				3	6											9	7. 7
Total developing tumors			4	24	21	7	8	4	3			1	1			73	8. 4

TABLE 3.—Experiment 300. Occurrence of interstitial-cell tumors of testes in BALB/c mice implanted with 10 percent stilbestrol-cholesterol pellets at 2 months of age—pellets removed as soon as testes reached 9 to 10 mm. in length

Mice	Age in months: 2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Total	Mean age (*months*)
Total	94	86	84	84	83	82	82	82	82	82	82	81	81	71	66		
Dying without tumor		8	2		1							1		10	5	27	12. 4
Tumor in right testis			1	7	4	6	6	1	3	2						30	9. 1
Tumor in left testis			1	6	6	5	8	1	1	2		2	1	1		34	9. 8
Simultaneous bilateral tumors					2		1									3	8. 7
Total developing tumors			2	13	12	11	15	2	4	4		2	1	1		67	9. 4

As in other types of tumors, it was impossible to know precisely when a testicular tumor originated. Hence a uniform, reproducible, albeit arbitrary, criterion for the presence of a tumor was adopted.

With this criterion for the appearance of tumors, tables 1 to 3 and text-figure 1 show that the 2 groups of mice that received 20 percent stilbestrol pellets (expts. 317 and 300A) had closely similar results in terms of the cumulative frequency of tumors and the time of their appearance. The cumulative frequency recorded for 300A, of 87 percent, is somewhat lower than the 98 percent for experiment 317, but the mean time (8.4 and 8.7 months, respectively), and the median time (approximately 7.7 months) of appearance are almost identical. The proportion of bilateral tumors is somewhat higher in experiment 317, but the difference between 317 and 300A is not statistically significant.

The group implanted with 10 percent pellets showed a lower eventual frequency of tumors (81%), and the time of appearance was somewhat longer (mean, 9.4 months; median, 9.2 months). There were fewer simultaneous bilateral tumors; the difference between the frequencies in experiment 317 and 300 is statistically significant ($P<0.01$).

A greater number of tumors appeared in the right than in the left testis in experiments 317 and 300A, though only in the latter experiment is the preponderance of right-sided tumors of statistical significance ($P = 0.01$).

Growth Patterns of Tumors

In experiment 317, observations were analyzed on the growth patterns of 347 tumors in 290 mice. Thirty-three mice that died within 2 months after the removal of the pellet were excluded from this portion of the study. Eight of these animals had bilateral tumors, so that 41 of the 388 gross tumors (323 plus 65) listed in table 1 do not appear in table 4.

Consideration of the growth graphs showed that independent observers could divide the course of the tumors into the following 7 types, with less than 10 percent of disagreement in regard to the arbitrary classification that was devised:

I. *Progressive growth.* These tumors continued to enlarge after removal of the pellet, with only minor fluctuations in the rate of progressive enlargement.

II. *Persistent-progressive growth.* For several months after the pellets were removed, these tumors remained stationary in size, or even became smaller without reaching the normal size of 6 to 7 mm., and then entered a period of progressive growth similar in rate to those of type I.

III. *Short regression-progressive growth.* After removal of pellets, these tumors regressed until the testes were of normal size, but resumed progressive growth within 2 months after reaching normal size. The 2-month period was selected arbitrarily, after experience had shown that regression usually lasted considerably longer than 2 months if growth was not resumed within that time.

IV. *Persistent or nongrowing tumors.* These masses remained at approximately the same size (9–11 mm.) during many months of observation, without either progressive growth or regression.

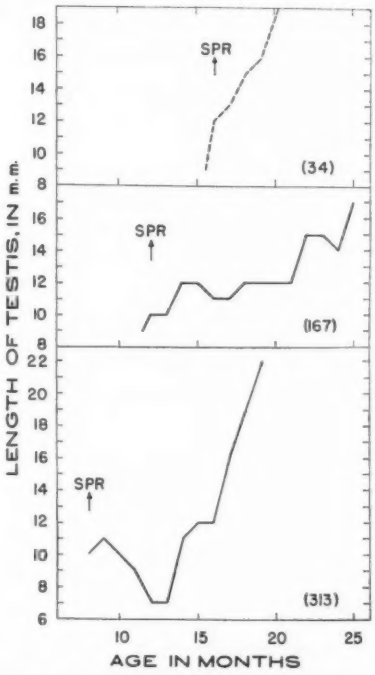
V. *Fluctuating-persistent tumors.* A few testes remained enlarged and showed marked irregular periods of increase and decrease in size, without commitment to either progression or regression for periods up to 20 months.

VI. *Regressed tumors.* These tumors, after either no increase or only slight increase in size, receded and the testes either returned to normal or became smaller and grossly atrophied and remained so for the subsequent period of observation.

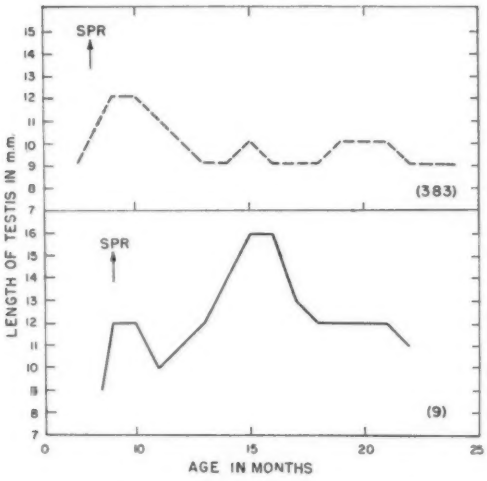
VII. *Regressed-late recurrent tumors.* These tumors, after the testes had regressed and remained normal for more than 2 months, resumed progressive growth.

These patterns could again be regrouped into growing tumors (types I, II, and III), persistent tumors (types IV and V), and regressed tumors (types VI and VII). The patterns are illustrated by specific examples in text-figures 2, 3, and 4.

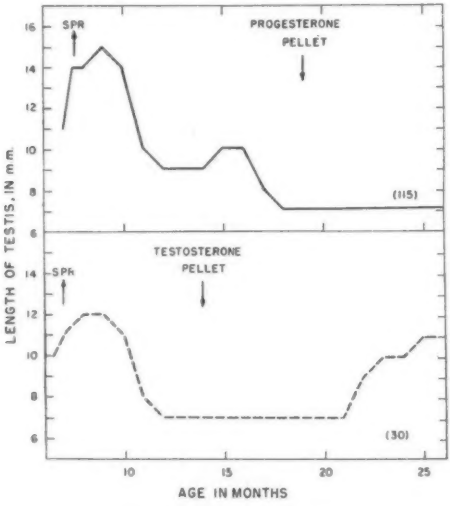
Of course these divisions are arbitrary, and all gradations between the examples shown were observed. The classification of a tumor is affected



TEXT-FIGURE 2.—Growth patterns of testicular tumors: growing tumors.
Solid line, right testis; *dashed line,* left testis.
Numbers in parentheses refer to individual mice of experiment 317; "SPR" marks time stilbestrol pellet was removed.
Upper panel: progressive growth (type I).
Middle panel: persistent-progressive growth (type II).
Lower panel: short regression-progressive growth (type III).



TEXT-FIGURE 3.—Growth patterns of testicular tumors: persistent tumors.
Solid line, right testis; *dashed line*, left testis.
Numbers in parentheses refer to individual mice of experiment 317.
"SPR" marks time stilbestrol pellet was removed.
Upper panel: persistent or nongrowing (type IV).
Lower panel: fluctuating-persistent (type V).



TEXT-FIGURE 4.—Growth patterns of testicular tumors: regressed tumors.
Solid line, right testis; *dashed line*, left testis.
Numbers in parentheses refer to individual mice of experiment 317.
"SPR" marks time stilbestrol pellet was removed.
Upper panel: regressed (type VI).
Lower panel: regressed-late recurrent (type VII).

by the period of observation; a mouse living longer after regression of its tumor has a greater opportunity of having a recurrence. The chief point to be made is the great variability in the courses of these neoplasms.

Gardner (*4*) studied the growth patterns of ovarian tumors procured by transplanting the ovaries of mice into the spleen.[4] He divided the growth patterns into 5 types, 4 including tumors that grew rapidly or slowly and 1 including those that grew slowly or remained unchanged over long periods. He also described 5 tumors that "either disappeared or were of microscopic size at autopsy." The charts in Gardner's paper and those included here are similar. Gardner concluded from his study of ovarian tumors that "their progression is erratic and variable."

Table 4 indicates the distribution of the tumors according to the type of growth pattern. In mice in which the stilbestrol pellet was removed 2 weeks after the testes were 9 to 10 mm. in length, 41 percent of the tumors continued to grow progressively (types I, II, and III), 16 percent persisted (types IV and V) but did not demonstrate progressive growth during an average observation period of approximately 9 months, and 43 percent regressed to the size of a normal testis for over 2 months (types VI and VII).

Of the 151 regressed tumors, 49 recurred eventually. Approximately half of the recurrences of these 49 tumors became evident within 4 months after regression, but in 6 instances recurrence took place 9 to 12 months after the testes had receded to normal (or smaller) size. Histologic sections from 4 of these latter testes showed extensive fibrosis, atrophy, other degenerative changes, and a mass of interstitial tumor tissue indistinguishable from tumors that had grown progressively.

The length of time necessary for the initial regression of a tumor did not appear to be correlated to the length of subsequent regression or to the tendency for late recurrence.

The mechanism of recurrence was clarified by histologic observations. Testes that had contained a gross tumor but had returned to normal size after the removal of the estrogen pellet showed extensive fibrotic, atrophic changes, with calcification of the capsule, cholesterol slits, and blood pigment. In the midst of these retrogressive manifestations, however, there were often microscopic, viable-appearing nodular areas of interstitial cells that could not be distinguished from obvious, large tumor masses. This persistence of viable tumor nodules in a sclerotic, atrophic remnant of a testis is illustrated in figures 1, 2, 3, and 4.

Among the 102 mice recorded in table 4 as having regressed tumors, histologic sections were obtained of 30 testes that had remained regressed for 8 to 17 months after the removal of the stilbestrol pellet. All these testes showed typical fibrotic changes, but 16 of the 30 also contained definite microscopic nodules or foci of interstitial cells. Presumably, at least some of these microscopic tumors could have resumed growth and recurred as a gross tumor.

Inspection of table 4 also shows that there is no relation between the

[4] Our attention was called to this study after completion of our analyses.

TABLE 4.—Growth patterns of 347 testicular tumors of experiment 317, by months of appearance

Type of growth	Age in months: 5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Total	Mean observation period (*months*)
I. Progressive	2	5	11	10	11	3	2	5	2		1		1			53	4. 7
II. Persistent-progressive		3	11	13	15	3	8	1	5	1	2					62	8. 7
III. Short regression-progressive	3	3	7	8	1	1	2				1					26	11. 1
IV. Persistent			4	5	4	1	2	1	1				1			19	9. 0
V. Fluctuating-persistent	1	1	7	8	6	3	4	4	1	1						36	8. 4
VI. Regressed	2	12	15	32	19	7	4	3	1	2		1	2	1	1	102	11. 3
VII. Regressed-late recurrent	2	3	10	26	2	4		1	1							49	14. 2

age of appearance of testicular tumors and their growth patterns. This would indicate that the growth characteristics of an established interstitial-cell tumor are independent of whether the host is relatively young or old, and, at the same time, independent of whether the tumor arose after relatively short or relatively long periods of continued estrogenic stimulation.

Bilateral Tumors

The occurrence of tumors in both testes provided one of the more interesting aspects of the work. In experiment 317, bilateral tumors in 57 of the 65 mice were followed in the study of growth patterns and supplied additional evidence of the remarkable individuality of tumors growing in the same host.

All combinations of growth patterns listed in table 4 were observed: In 2 mice, both tumors grew progressively; in 3, both persisted; in 7, both regressed; in 3, both regressed and then recurred (*see upper panel* text-fig. 5). Thus, similar behavior of both tumors occurred in 15 of 57 mice, or 26 percent of the animals.

Bilateral tumors that showed similar behavior for the first part of their course, and different behavior for the remainder, arose in 12, or 21 percent, of the mice. In 9 mice, both tumors receded to normal size and one recurred while the other did not. The middle panel of text-figure 5 illustrates a situation in which one tumor persisted and the other regressed for the initial period of observation; both resumed progressive growth, but this started at 18 months in the left testis and at 22 months in the right.

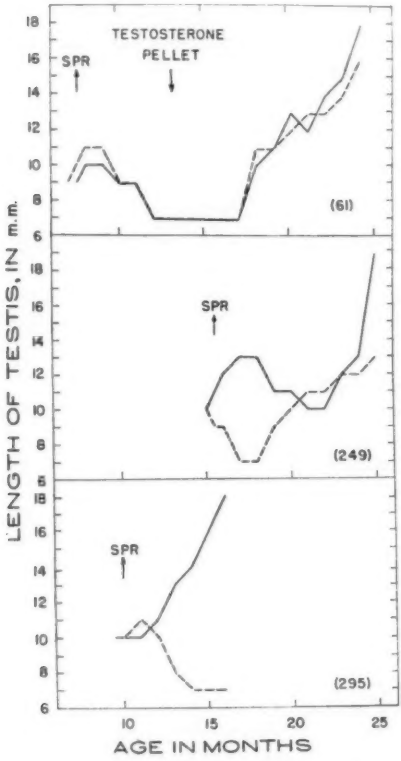
In 30 mice, or 53 percent, the 2 testes showed different patterns of growth. In 15 mice, 1 tumor grew progressively and the other regressed; this is illustrated in the lower panel of text-figure 5. In 10 mice, 1 tumor grew progressively and the other persisted without definite progression. In 5 animals, 1 tumor regressed and the other persisted.

It should be pointed out that the majority of animals in which only one testis became grossly enlarged really represents even greater divergence of response in paired glands exposed to an identical stimulus. This is an impressive demonstration of local influences upon tumor development and growth.

The discussion of bilateral testicular tumors in this section has been limited to instances of tumors which appeared at approximately the same time in one animal. In experiment 317 there were also 32 instances of tumors appearing in the second testis at varying periods after the removal of the estrogen pellet. These tumors have not been recorded in table 4, since they represent a somewhat different problem. However, these nonsimultaneous bilateral tumors showed growth patterns similar to those listed in table 4.

Effect of Testosterone and Progesterone on Regressed Tumors

In earlier work (*2*) it was found that prompt recurrence of a regressed testicular tumor could be achieved in a high proportion of mice upon the



TEXT-FIGURE 5.—Growth patterns of bilateral testicular tumors. *Solid lines*, right testis; *dashed lines*, left testis. Numbers in parentheses refer to individual mice of experiment 317. "SPR" marks time stilbestrol pellet was removed. *Upper panel*: similar growth of both tumors. *Middle panel:* dissimilar growth for a period, followed by similar growth pattern by both tumors. *Lower panel:* dissimilar growth of both tumors.

reimplantation of another stilbestrol pellet. One of the purposes of experiment 317 was to study the effect of other hormonal stimuli upon the recurrence of these neoplasms. It was postulated that perhaps regression could be maintained in a higher proportion of animals if they were treated with testosterone or progesterone.

Therefore, mice that showed regression of the tumors for over 2 months were entered into 3 groups: One was implanted subcutaneously with solid pellets of testosterone or testosterone propionate, weighing 5 mg.; the second was implanted with a solid progesterone pellet weighing 15 mg.; the third represented the untreated controls.

Table 5 shows that these procedures had no effect on the subsequent course of the tumors. The mice that received testosterone, in fact, had a higher proportion of late recurrences, though the difference between this group and the control is not statistically significant. Much more

TABLE 5.—Lack of effect of testosterone and progesterone on estrogen-induced interstitial-cell testicular tumors that had regressed for a minimum of 2 months after removal of stilbestrol pellets—testosterone pellets, 5 mg. each, and progesterone pellets, 15 mg. each, implanted subcutaneously

	Mice with regressed tumor (*number*)	Tumor remained regressed (VI)	Tumor showed late recurrence (VII)
Control (no pellet)	56 (100)*	34 (61)	22 (39)
Testosterone	40 (100)	20 (50)	20 (50)
Progesterone	27 (100)	17 (63)	10 (37)

*Numbers in parentheses are percent. The differences in distribution of the 3 groups are not significant ($P > 0.2$ for largest difference).

work is essential to establish that the recurrence of these tumors cannot be inhibited. More potent hormones and combinations of hormones should be explored. This analysis of the present experiment, showing no significant effects under the conditions used, allowed all animals in the three groups to be considered together in other discussions of the paper.

Effect of Short Additional Estrogenic Stimulation

In experiment 317, stilbestrol pellets were removed 2 weeks after the enlargement of the testes to between 9 and 10 mm. in length; when the pellets were removed, the testes were 9 to 16 mm. long, with an average of 11.0 mm. In contrast, mice of experiments 300 and 300A had their pellets excised as soon as the testes reached 9 to 10 mm. (average: = 9.6 mm.). Thus, mice of experiment 317 had an additional period of estrogenic stimulation at a specific phase in the growth of their tumors.

The growth of tumors in experiments 300 and 300A was compared with the patterns observed in experiment 317. After the animals that were followed for less than 2 months were discarded, and when simultaneous bilateral tumors were considered as 2 individual tumors, analyses were made on 67 of 82 tumors in the 101 mice that received 20 percent pellets, and on 59 of 70 tumors in the 94 mice that were implanted with 10 percent pellets. Classification of the tumors into 7 types divided the available numbers into groups that were too small, and they were regrouped into the 3 major types: progressively growing, persistent, and regressing.

Table 6 presents the results and shows clearly that there was no difference in the distribution in the growth patterns between experiments 300 and 300A, whereas there was a highly significant difference between these two groups and experiment 317. The proportion of progressively growing tumors was doubled in the mice receiving the short period of additional estrogenic stimulation after their testes reached 9 to 10 mm. in length. Conversely, a significantly smaller proportion of the tumors regressed.

The results show that a relatively short stimulus at a specific time of growth had a profound effect in shoving these tumors from the dependent

TABLE 6.—Effect of hormonal stimulation on growth pattern of interstitial-cell testicular tumors*

	Total tumors	Progressive growth (I, II, III)	Persistent growth (IV, V)	Regression † (VI, VII)
Ten percent stilbestrol pellet removed when testes reached 9 to 10 mm. (expt. 300)	59 (100)	12 (20)	6 (10)	41 (70)
Twenty percent stilbestrol pellet removed when testes reached 9 to 10 mm. (expt. 300A)	72 (100)	9 (12)	12 (17)	51 (71)
Twenty percent stilbestrol pellet removed *2 weeks* after testes reached 9 to 10 mm. (expt. 317)	347 (100)	141 (41)	55 (16)	151 (43)

*Numbers in parentheses are percent: Forty-one percent of 347 versus 12 percent of 72 or 20 percent of 59 is significant ($P < 0.01$). Forty-three percent of 347 versus 71 percent of 72 or 70 percent of 59 is significant ($P < 0.01$).
†Of the 41, 51, and 151 regressed tumors, 5, 4, and 49, respectively, recurred.

to an autonomous phase. The effect was not due simply to a greater dose or longer period over which estrogen acted, since the distribution of growth patterns was the same in mice that received 10 percent and 20 percent pellets and was the same among tumors that appeared earlier than the average time of induction as among those that appeared later (expt. 317).

These studies clearly indicate that, at least for the interstitial-cell testicular tumor induced by estrogen, the transition from the dependent to the autonomous behavior is neither regular nor uniform. Not only is there great variation in this sequence of events but also it seems to be not one-directional. The best description that occurs to us is that the tumor represents a biologically unstable system.

DISCUSSION

The results reported here extended earlier findings attending the removal of stilbestrol-cholesterol pellets from BALB/c mice after the mice had developed testicular tumors. A lengthy discussion is unnecessary because pertinent comments are presented along with the experimental findings where they are more appropriate. Two aspects of the work are reviewed briefly:

First, in the previous study (*2*) the pellets were removed at different times after tumors appeared and the testes were measured at monthly intervals. While this procedure revealed pronounced variations in the response of tumors to the termination of estrogenic stimulation, the response could only be listed as tumors that either grew or regressed and, of the latter, those that recurred.

Experiment 317, described herein, was an effort to obtain more information along these lines. A criterion for the time of appearance of a testicular tumor was adopted, the pellets were removed 14 days later,

and the testes were measured at weekly intervals. With this more uniform and detailed procedure, the growth of each testis was plotted on graph paper and the patterns were analyzed. The growth patterns could be divided into the 7 types listed in table 4 and shown in text-figures 2 to 4. The original group of tumors that grew was subdivided into 3 types; a new group of persistent tumors included 2 types; the original group of regressed tumors was divided into 2 types, one of which included the recurrent tumors.

These same growth patterns emerged when bilateral tumors were plotted, regardless of whether the tumors arose simultaneously or at varying periods in the same animal. Thus the extraordinary individuality of induced testicular tumors in the mouse was again emphasized. The tumors exhibit every conceivable variation in growth patterns ranging from rapid progressive growth to permanent regression, or extended periods of regression followed by recurrence. As stated previously (*2*), they can be used to advantage in studies on tumor progression, especially those involving hormone dependency. At present it seems impossible to formulate a simple expression of their growth characteristics other than that they represent a biologically unstable system.

Huseby (*5*, *6*) has used testicular tumors to advantage in his studies on hormone dependency and hormone production. His results show that these characteristics also are as variable in the transplanted tumors as our growth patterns are in the original tumors. For example, although most tumors on transplantation display decreasing dependency to estrogen, at least one serially transplanted tumor increased in dependency as transplantation progressed. Also, although many of the tumors produce androgen, some lines release significant amounts of progestational and estrogenic hormones. There appears to be no absolute correlation between the production of hormones by these tumors and the degree of dependency, the growth rate, or their morphologic appearance.

It has been known for many years that tumors undergo changes in growth properties when transplanted serially into suitable hosts (*7*). Tumors arising spontaneously in the same animal may not grow at the same rate, and geneticists (*8*) have shown they may differ in the number of genes controlling transplantability. Further, an abrupt change in growth characteristics is usually accompanied by a change in the number of genetic factors. Foulds (*9*) has shown that certain mammary tumors of mice are hormone responsive in that they grow only during pregnancy and regress after parturition. Most previous investigations with tumors showing different growth patterns were carried out with transplanted tumors; the spontaneous mammary tumors described by Foulds were those arising from "plaques" within the breast tissue instead of the usual "hyperplastic nodules." Mammary tumors of mice possess different histologic characteristics (*10*) as well as biologic properties. In contrast, interstitial-cell tumors of the testes of mice show a common histologic pattern and, according to the data presented to date, a wider variation in growth patterns. Furthermore, the present results show that the

wide divergence in growth patterns is displayed by the hosts in which they arise. They should prove useful in further studies of hormone-dependent primary tumors.

The second aspect of interest was the striking influence that a brief, additional exposure to estrogenic stimulation exerted upon the biologic properties of the induced tumors. In experiment 300A, pellets were removed from mice as soon as their testes were 9 to 10 mm. in length, and 12 percent of the tumors grew progressively, 12 percent persisted, and 71 percent regressed, of which 8.5 percent recurred. In experiment 317, when pellets were removed 14 days *after* the testes measured 9 to 10 mm., 41 percent of the tumors grew progressively, 16 percent persisted, and 43 percent regressed, of which 32 percent recurred. It is clear that in mice exposed to only 2 weeks of additional stimulation more tumors grew and fewer regressed. Also, more of the regressed tumors recurred (table 6).

This effect of an additional 2 weeks of exposure to stilbestrol at a specific phase of the growth of the tumors should be related to the fate of tumors arising after the removal of the stimulus and to tumors in mice in which the stimulus is retained. In a previous experiment (*3*) BALB/c mice received stilbestrol-cholesterol pellets and these were excised 16 to 24 weeks later, or before the testes became grossly enlarged. Twenty-eight percent of the mice eventually developed testicular tumors, at an average age of 15 months, or long after the removal of the stimulus. Of these tumors, 48 percent grew progressively, 45 percent grew to a limited size and then persisted without progression, and only 7 percent regressed. This is in contrast with regression of 71 percent of the tumors in mice of experiment 300A, in which the stilbestrol pellet was removed as soon as a gross tumor was detected. The greater frequency of regression of tumors that arose on continued estrogen stimulation is also evident, if the absolute numbers rather than relative distribution values are used. Such regression occurred in 51 of 101 mice that carried pellets until tumors appeared, in contrast with only 7 of 359 mice that had the pellets removed before the tumors became evident. These results indicate that tumors developing in the presence of estrogen are more dependent upon it for progressive growth than those occurring in mice after the pellets are removed.

It is known that other tumors dependent upon hormonal stimulation for their occurrence can develop after the stimulus has been removed. For example, mammary tumors occur in mice that have been ovariectomized at an early age (*11*). The data presented here show that limited hormonal stimulation not only induces testicular tumors but also alters the biologic properties of the tumors.

Finally, this study confirmed earlier findings that the tendency to spontaneous recurrences was evidence of lasting changes in the testes and, as before (*2*), microscopic examination of testes, in which tumors had regressed or in which no tumor had appeared during estrogenic stimulation, confirmed the presence of persisting nodules of interstitial cells. It

also supplied further evidence that the tumors are the end product of a gradual process resulting from the interplay of extrinsic factors and intrinsic host factors.

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PLATE 123

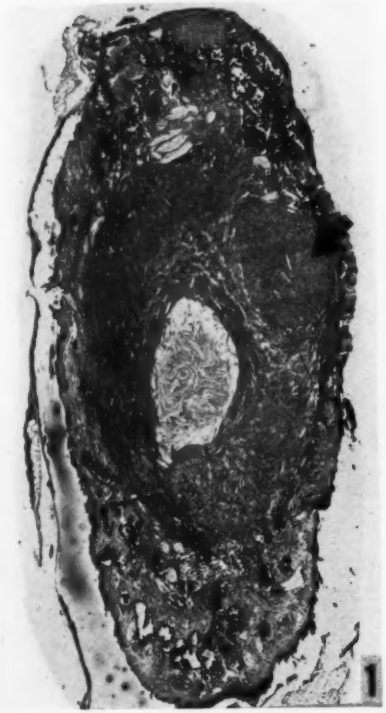
Testis of BALB/c mouse implanted subcutaneously with a 20 percent stilbestrol-cholesterol pellet at 2 months of age. Right testis became enlarged 12.5 months later, when pellet was removed. Testis returned to normal size during following 2.5 months, and remained small for 7 months. Mouse died at 2 years of age.

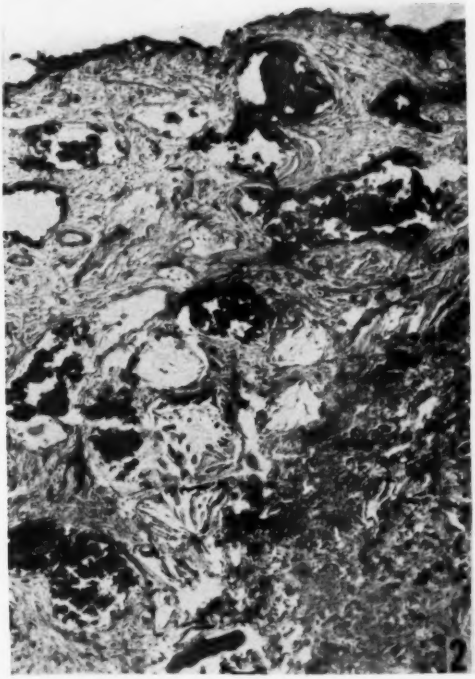
FIGURE 1.—Cross section of testis, showing atrophy and fibrosis, with a nodule of viable interstitial cells. Hematoxylin and eosin. × 15

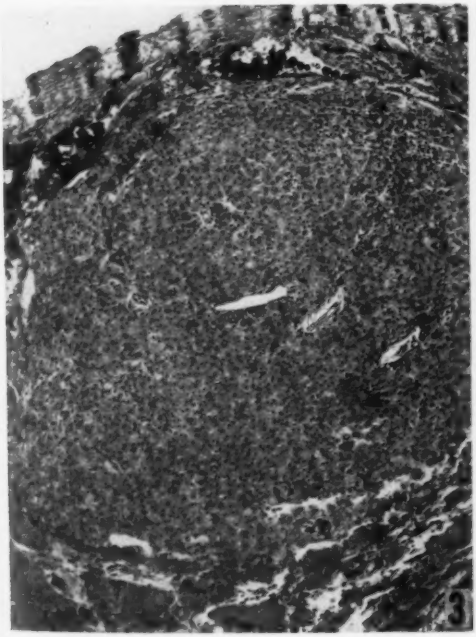
FIGURE 2.—Fibrosis and calcification near capsule of testis. × 160

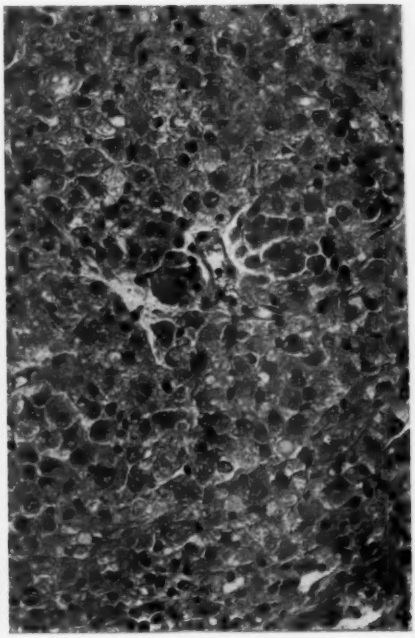
FIGURE 3.—Interstitial-cell nodule in testis. × 160

FIGURE 4.—Higher-power magnification of the interstitial-cell nodule. × 400









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Dr. Floyd C. Turner

1889–1960

The death of Dr. Floyd C. Turner, Senior Surgeon, USPHS (Ret.), on March 5, 1960, took from the field of cancer chemotherapy a pioneer researcher and one of its most devoted workers. Dr. Turner first became interested in this field of research while attached to the USPHS Cancer Investigations Unit located at Harvard Medical School, Boston. His interest was increased by the untimely death of his wife from cancer. Dr. Turner was detailed to the Boston unit in 1936 and became its Executive Officer in 1937, upon the retirement of Dr. J. W. Schereschewsky who had directed it from the time it was established.

This unit and another one located at the National Institute of Health in Washington, D.C., were consolidated in 1937 to form the staff of the new National Cancer Institute. When both units were moved to the Institute's new building at Bethesda, Maryland, in 1939, Dr. Turner became the first Executive Officer of the Institute's laboratories under its first Chief, Dr. Carl Voegtlin. He continued his research and was the first cancer investigator to demonstrate that malignant tumors could be produced in rats by the use of a plastic. (Turner, Floyd C. : Sarcomas at sites of subcutaneously implanted Bakelite disks in rats. J. Nat. Cancer Inst. 2: 81–83, 1941.)

After his retirement in 1945, Dr. Turner lived in Boulder Creek, California, where he continued his research in cancer chemotherapy in a small laboratory which he built and partially maintained at his own expense. His work was aided by grants from the National Cancer Institute. His studies were concerned chiefly with the effects of bacterial filtrates and a wide variety of chemicals on malignant tumors in laboratory animals.

Dr. Turner was born in Lincoln, Kansas, December 28, 1889. He received his early education in schools in that State and in South Dakota. After a four-year tour of duty as a hospital corpsman in the Navy he studied medicine and received his M.D. degree in 1916 from the Chicago College of Medicine and Surgery, later incorporated into the Loyola University School of Medicine.

Immediately after the entrance of the United States into World War I, Dr. Turner volunteered and was commissioned in the Medical Corps of the U.S. Army. He served throughout the War. He became a Commissioned Officer of the Public Health Service in September, 1920. His service included three years with the American Consulate in Belfast, Ireland, and several years as Officer in Charge of the Marine Hospital, Buffalo, New York. He was transferred to the National Institute of Health in 1932.

The exigencies of war took Dr. Turner away from his beloved research in April, 1942, when he was transferred to the New York Quarantine Station as Executive Officer. The heavy wartime duties of this position proved to be too

great a strain for an already impaired heart, and he had to retire on disability in early 1945.

Dr. Turner's entire life was one of service to his country and his fellow men. His excellent work in every position he held earned for him the high regard of his superior officers and associates.

Dr. Turner is survived by his wife, the former Mrs. Elna Graff, whom he married in 1953, and by three daughters by his first marriage, Mrs. Velma Robinson, Lafayette, California, Mrs. Virginia Welton, Orange, California, and Mrs. Lillian Erst, Bridgeport, Connecticut.

Ora Marshino

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"Histopathology of tumors induced in mice using polyoxyethylene sorbitan monostearate," by Giuseppe Della Porta, Benedetto Terracine, Kai Dammert, and Philippe Shubik.

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